Non-tuberculous Mycobacteria in The Gambia: Prevalent Species, Carriage and Disease

Thesis

Non-tuberculous Mycobacteria in The Gambia: Prevalent Species, Carriage and Disease

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Abstract

Pulmonary tuberculosis-like disease caused by non-tuberculous mycobacteria (NTM) has gained attention, in part, because of its increased recognition especially in the elderly and immunocompetent population. This is an emerging problem of public health significance. With the increasing incidence of case reports and series from diverse countries and regions of the world, the distribution of NTM species isolated from clinical samples appear to vary significantly by region. However, very little is known about the contribution(s) of NTM to tuberculosis-like disease, and significant knowledge gaps exist regarding their geographical distribution, clinical and molecular epidemiology in low and middle income countries (LMIC) where there is a high burden of disease caused by *Mycobacterium tuberculosis* complex (MTBC). This dissertation provides a state of the art review of the current epidemiology of NTM carriage and disease in sub-Saharan Africa and presents results from a study of the epidemiology of NTM in The Gambia on pulmonary carriage and disease.

I systematically searched electronic databases (PubMed, Embase, Popline, Ovid and Africa Wide Information) for English language articles on pulmonary NTM in sub-Saharan Africa published from Jan 1,1940 to Oct 1, 2016. The American Thoracic Society and Infectious Disease Society of America (ATS/IDSA) diagnostic criteria was applied to differentiate between colonisation of NTM and clinically relevant pulmonary NTM disease. *Mycobacterium avium* complex (MAC) species were the most frequently isolated NTM (15.0% - 57.8%) in colonisation while *M. kansasii*, was the most common (184 [69.2%] of 266) cause of confirmed pulmonary NTM disease. A significant proportion (2,623 [29.2%] of 8,980) of NTM isolates in the studies reviewed were not identified to species level. This and the absence of detailed clinical and radiological data hampered the assessment of the clinical relevance of all NTM isolates.

A nationwide Tuberculosis prevalence survey provided a platform for me to investigate the population prevalence and molecular epidemiology of NTM in pulmonary samples in the general population of the Gambia. Here, I confirmed suspected NTM cultures from decontaminated sputa stored from the parent survey by *16S rRNA* gene sequencing analysis and applied the ATS/IDSA diagnostic criteria to determine clinical relevance of identified NTM. The prevalence of NTM in pulmonary samples was 39.8% [95% Confidence Interval, CI: 35.8% – 44.0%). *M. avium* complex was by far the most commonly isolated NTM (71.0%), followed by *M. fortuitum* (9.5%) and *M. nonchromogenicum* (2.9%). All age groups were three times as likely to have NTM in their sputa as the 15-24 age group (p= 0.017), while urban compared to rural residents were 40 times less likely to be NTM positive (p =0.012).

Collectively, these findings highlight the contribution of NTM to colonisation and the risk for over diagnosis of smear positive TB, given the prevalence in presumptive TB cases in The Gambia. I have highlighted the knowledge gap resulting from incomplete identification of NTM species in the sub-continent and the risk of misdiagnosis of pulmonary NTM as PTB when smear microscopy is the sole diagnostic modality. Additional research and surveillance is therefore required to investigate the full contribution of NTM to pulmonary disease, particularly in high risk groups and it is important to review the existing tuberculosis identification methods for presumptive tuberculosis suspects in The Gambia, and sub-continent.

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Dedication

This thesis is dedicated to my mother Mrs Cecilia Manka Catherine Young. Thank you Mummy for always being there for me

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Abbreviations

16S rRNA	16S ribosomal Ribonucleic Acid
AAFB	Acid and Alcohol Fast Bacilli
AIDS	Acquired Immune Deficiency Syndrome
ATCC	American Type Culture Collection
ATS/IDSA	American Thoracic Society/ Infectious Diseases Society of America
BLAST	Basic Local Alignment Tool
bp	Base pair
C	Cytosine
CF	Cystic Fibrosis
CFP-10	Culture Filtrate Protein-10
COPD	Chronic Obstructive Pulmonary Disease
CXR	Chest X Ray

DNA	Deoxyribonucleic Acid
ESAT-6	Early Secretary Antigen Target-6
EXOSAP	Exonuclease Shrimp Alkaline Phosphatase
GAMSTEP	Gambian Survey of Tuberculosis Prevalence
H_2O	Water
HIV	Human Immunodeficiency Virus
hsp	Heat Shock Protein
KEMRI	Kenya Medical Research Institute
MAC	*Mycobacterium avium* complex
MGIT	Mycobacteria Growth Indicator Tube
mL	Milliliter
mM	Millimolar
MPT64	*Mycobacterium tuberculosis* protein 64
MRCG	Medical Research Council Unit The Gambia
MSMD	Mendelian Susceptibility to Mycobacterial Diseases
MTBC	*Mycobacterium tuberculosis* complex
NALC	N-acetyl-L-cysteine
NCBI	National Centre for Biotechnology Information
NCTC	National Collection of Type Cultures

NTC	Non-template control
NTM	Non-tuberculous mycobacteria
P	Primer
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
rpoB	RNA polymerase B
SCC	Scientific Coordinating Committee
sSA	Sub-Saharan Africa
TAE	Tris-acetate ethylene diamine tetra acetic acid
TB	Tuberculosis
TE	Tris ethylene diamine tetra acetic acid
U	Units
USA	United States of America
ZN	Ziehl Neelsen
µL	Microlitre
µM	Micromolar

Publications

Publications from this thesis

1. **Okoi C**, T B Anderson S, Antonio M, Mulwa SN, Gehre F, M O Adetifa I. Non-tuberculous Mycobacteria isolated from Pulmonary samples in sub-Saharan Africa - A Systematic Review and Meta Analyses. Sci Rep. 2017 Sep 20;7(1):12002. doi: 10.1038/s41598-017-12175-z. PubMed PMID: 28931915.
2. **Okoi C,** Ifedayo M.O, Suzanne T.B Anderson, Florian Gehre, Martin Antonio. "Non-tuberculous mycobacteria in the Gambia - prevalent species, carriage and disease." Manuscript in preparation.

Other relevant publications

1. Ofori Anyinam, Kanuteh F, Agbla FC, Adetifa I, **Okoi Catherine**, Dolganov G, Schoolnik G, Secka O, Antonio M, de Jong BC, Gehre F. **"**Impact of the Mycobacterium africanum West Africa 2 lineage on TB Diagnostics in West Africa - decreased sensitivity of rapid identification tests in The Gambia**.** PLoS Negl Trop Dis. 2016 Jul 7;10(7):e0004801. doi: 10.1371/journal.pntd.0004801. eCollection 2016.
2. Ifedayo MO Adetifa, Lindsay Kendall, Adedapo Bashorun, Christopher Linda, Semeeh Omoleke, David Jeffries, Rahmatulai Maane, Beatrice Dei Alorse, William Dei Alorse, **Catherine Bi Okoi,** Kodjovi D Mlaga, Ma Ansu Kinteh, Simon Donkor, Bouke C de Jong, Martin Antonio & Umberto d'Alessandro "A tuberculosis nationwide prevalence survey in Gambia, 2012.**"** Bull World Health Organ. 2016 Jun 1;94(6):433-41. doi: 10.2471/BLT.14.151670. Epub 2016 Apr 21.

1 Introduction

1.1 Non-tuberculous mycobacteria

Non-tuberculous mycobacteria (NTM) is a designation used for many mycobacterial species other than the following constituent members of *Mycobacterium tuberculosis* complex (MTBC) i.e *M. tuberculosis, M. bovis, M. africanum, M. canetti, M. microti, M. pinnipedii*, and *M. caprae*. Of the >150 NTM species reported in the literature, 25 species have been strongly associated with diseases; the remainder are environmental organisms rarely encountered in clinical samples [1]. Interest in NTM gained traction following the onset of the Human Immunodeficiency Virus/Acquired Immunodeficiency Syndrome (HIV/AIDS) epidemic. However, what is known of these organisms is mostly from studies/surveillance conducted in western and other high-income countries and very rarely from developing countries [2, 3].

NTM were identified soon after Koch's identification of *M. tuberculosis* as the cause of active TB in 1882. While many NTM species rarely if ever, cause disease, a few are implicated in disease and death. They are responsible for a wide array of clinical manifestation ranging from pulmonary (the most frequent), to lymph node (lymphadenitis, most common in children) and skin/soft tissue disease. Disseminated disease tends to occur among immunocompromised hosts and patients with single gene defects resulting in Mendelian susceptibility to mycobacterial diseases (MSMD) [4, 5]. For instance, *Mycobacterium avium* complex (MAC) disease can be fatal without adequate diagnosis and treatment in persons with HIV/AIDS [6].

Worldwide, there is now increasing recognition of pulmonary NTM disease in immune competent persons especially the elderly as a significant public health concern [2, 7-9].

However, a significant knowledge gap exists concerning the geographical distribution of NTM, and the prevalent species in carriage and disease in low and middle income countries (LMIC) where there is a high burden of active MTBC disease [2, 3, 10-13].

1.2 Biology of non-tuberculous mycobacteria

NTM are aerobic, slender, non-motile microorganisms and like MTBC, have the following characteristics:

(1) Are acid and alcohol fast bacilli (AAFB) i.e. give a positive reaction with acid alcohol fast stains.
(2) Have a guanine plus cytosine (G+C) content of deoxyribonucleic acid (DNA) of 61-71%.
(3) Have a thick lipid-rich, waxy cell wall that contains long chain fatty acids (C60-C90, mycolic acids), which on pyrolysis, are cleaved to C22 to C26 fatty acid methyl esters.
(4) Exhibit intracellular pathogenicity.

NTM include both slow growing (require 7 days or more to form colonies on subculture) and rapidly growing (form colonies on subculture in less than 7 days) species. In 1959, Runyon classified the NTM into four groups based on their rate of growth, production of pigment and whether this pigment is produced in the dark or only after exposure to light (Table 1).

Table 1: Runyon's classification of non-tuberculous mycobacteria

Category	Rate of growth	Description	Organism
Runyon 1 organisms (Photochromogens)	Slow growing	Produce a yellow-orange pigment when exposed to light	*M. kansasii, M. marinum, M. asiaticum, M. simiae*.
Runyon 2 organisms (Scotochromogens)	Slow growing	Produce a yellow-orange pigment regardless of whether they are grown in the dark or the light	*M.gordonae , M. scrofulaceum.*
Runyon 3 organisms (Nonchromogens)	Slow growing	Never produce pigment regardless of culture conditions	*M. avium* and *M. intracellulare* (together known as *Mycobacterium avium* complex), *M. ulcerans*
Runyon 4 organisms	Rapidly growing	Never produce pigment, regardless of culture conditions	*M. fortuitum, M. peregrinum, M. abscessus, M. chelonae, M. thermoresistible*

Slow growth of NTM is due to impermeability of the lipid-rich waxy cell wall and the synthetic energy cost of the long-chain mycolic acids. The extremely hydrophobic outer membrane of NTM predisposes them to biofilm formation in the environment and in tissue rather than growth in suspension. This tendency towards biofilm formation and replication in it, results in their preferential aerosolisation from water, [14] and increases the NTM resistance to disinfectants and antibiotics [15]. For example, in the post-surgical outbreak of *M. massiliense* that occurred in Brazil between 2006 and 2007, the clone BRA100

responsible for the epidemic was tolerant to the 2% glutaraldehyde used to disinfect surgical equipment [16].Thermal resistance is considered a major driver of NTM ecology. However, the molecular and genetic basis of this thermal resistance is poorly understood. Schulze - Robbecke *et al.* showed that it took 346 minutes at 55°C to kill 90% of *M. xenopi* cells - the most heat resistant NTM species [17]. Therefore, it is not surprising that *M. xenopi* outbreaks are often associated with water heating systems and NTM cells are frequently isolated from household water pipes where temperatures approach 50°C [18].

NTM cells are also relatively resistant to low pH exemplified by survival of very large numbers of *M. avium* complex cells in the acidic, brown-water swamps off the eastern coast of the United States of America (USA) [19] and survival by some MAC strains in the stomachs of persons living with HIV [20]. However, the majority of NTM have evolved to grow under conditions of moderate salinity (1 to 2% NaCl) except for *M. marinum,* a pathogen of salt-water fish in aquaria. There it grows in conditions of 3 to 4% NaCl, approaching the salinity of the ocean.

NTM survive and grow even better in amoebae and protozoa unlike many other bacteria that are consumed for carbon and energy. As a result, they are categorized as amoebae - resisting microorganisms (ARMS) [21]. It is hypothesized that amoebae and protozoan grazing resulted in selection for, and emergence of, NTM that can survive and colonise or cause disease in mammalian macrophages. This is supported by findings of *M. avium, M. kansasii, M. massiliense, M. marinum and M. fortuitum* strains surviving and actively replicating in *Acanthamoeba polyphaga, Acanthamoeba castellani* and in the ciliated protozoan *Tetrahymena pyriformis* [21].

For many years, the phenotypic and growth rate system of Runyon as described above was the only method of classifying these organisms. More recently, NTM phylogeny studies utilizing multigene sequencing of semi-conserved genes such as those for RNA polymerase B (*rpoB*), heat-shock protein (*hsp-65* KD) and 16S ribosomal ribonucleic acid (*16S rRNA*) have facilitated the identification of previously unknown species and led to a more extensive taxonomy [22, 23] as illustrated in Figure 1.

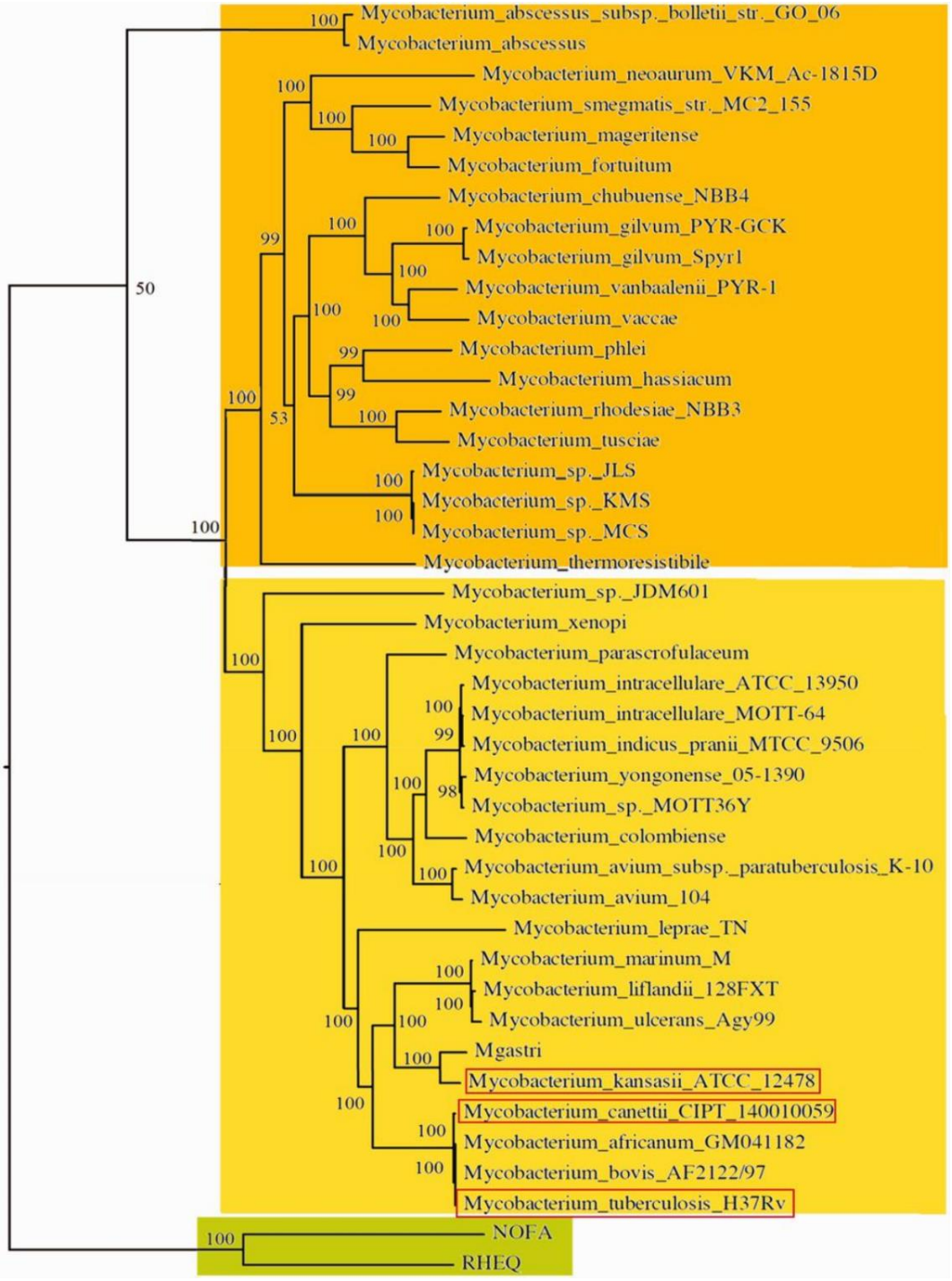

Figure 1: Phylogenetic relationships among Mycobacterium genus. The scale bar represents amino acid changes per site. Rapid growing species are shadowed in orange, slow growing species are shadowed in yellow, and outgroup species are shadowed in green. ***Mycobacterium tuberculosis***, ***M. canettii***, and ***M. kansasii*** are boxed in red [24].

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1.3 Epidemiology of non-tuberculous mycobacteria

1.3.1 Occurrence and geographical distribution

NTM are found worldwide [2, 13] and have been isolated from a variety of environmental sources including raw and treated water sources, water distribution systems, household plumbing systems, soils and animals [25, 26]. In the most comprehensive published description of the global distribution of NTM , *M. avium* complex (*M. avium and M. intracellulare*), *M. gordonae, M. chelonae, M. xenopi, M. fortuitum, M. abscessus, M. kansasii*, and *M. haemophilum* were the most frequently isolated NTM as shown in Figure 2. The prevalence of MAC, the most common NTM, ranged from a low of 31% in South America to a high of 71% in Australia. However, within the MAC species, significant continental/regional variability was noticed. For example, almost all MAC isolates from Australia and South Africa were *M. intracellulare* whereas *M. avium* was dominant in Europe, Asia, North America and South America. The distribution of *M. xenopi*, *M. kansasii* and rapidly growing mycobacteria (M. *fortuitum, M. abscessus, M. chelonae)* was also quite varied [13]. In addition, some NTM have been linked with specific environmental niches e.g. coastal regions. For example, *M. xenopi* is particularly prevalent in the regions of Croatia, Northern Italy, Ontario, Canada and in areas bordering the English Channel [8, 27]. *M. kansasii* is most frequently isolated in South America, Eastern Europe and the metropolitan centres of Paris, London, Tokyo and the Johannesburg region of South Africa. Urbanisation and commercial mining activities are major contributors to this observed distribution of *M. kansasii* [28, 29].

About 10-30% of all NTM isolates worldwide are rapidly growing NTM and they are most prevalent in Asia where they are associated with up to 50% of cases of pulmonary NTM

colonisation [12, 30-32]. *M. simiae*, which traditionally was confined to the southern United States, Cuba and Israel, is now emerging globally [13]. *M. malmoense, M. smegmatis, M. interjectum, M. scrofulaceum, M. peregrinum, M. colombiense,* and *M. nonchromogenicum* are rare and geographically restricted but are sporadically isolated from Europe, Asia, sub Saharan Africa, Southern and Northern America [2]. However, there is a dearth of data from sub-Saharan Africa which is also under-represented in this 'comprehensive' review of NTM epidemiology because the global NTM collection only had samples from two laboratories in South Africa making up about 28.0% (5646/20182) of the entire collection [13].

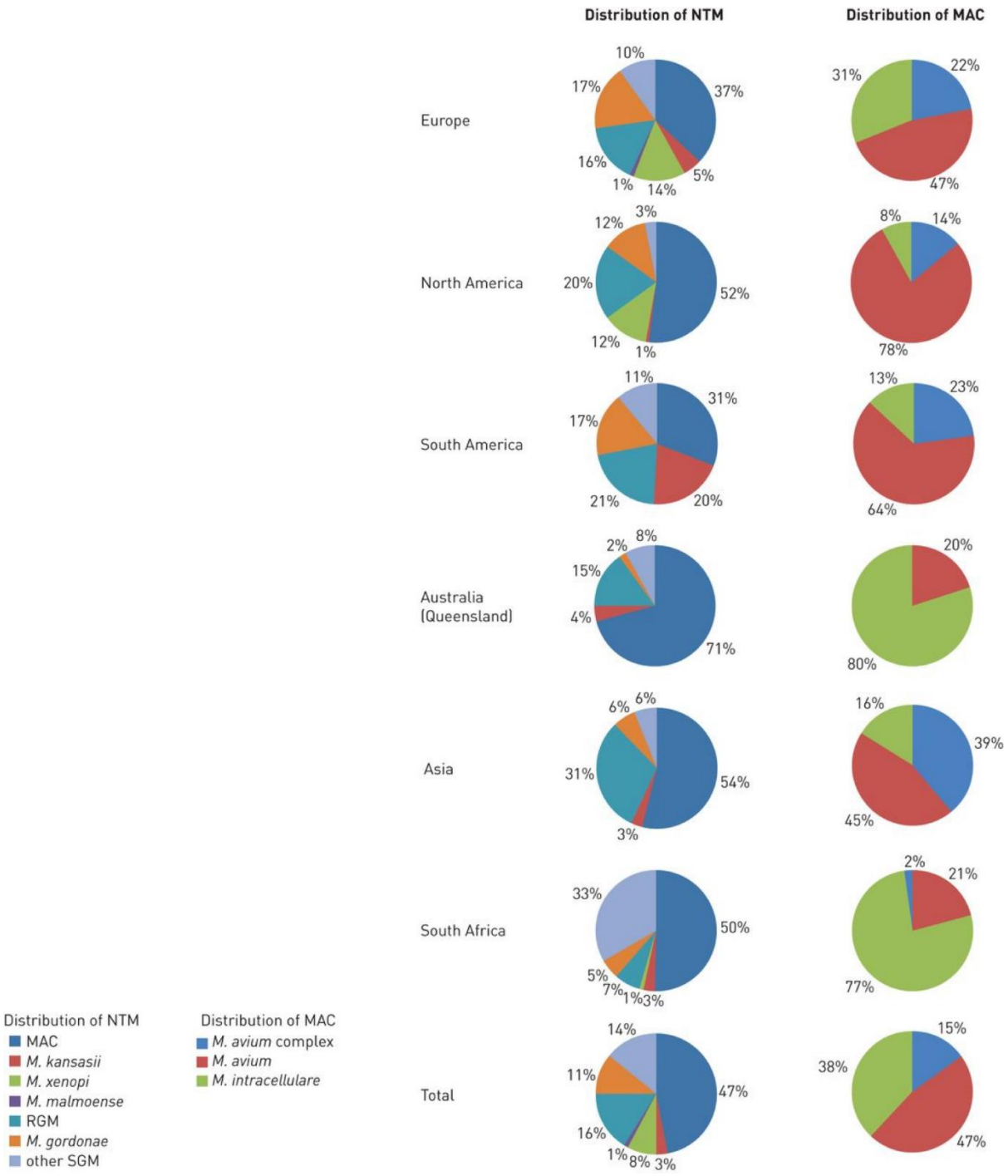

Figure 2: Worldwide distribution of different non-tuberculous mycobacteria from pulmonary samples in 2008. MAC: *M. avium* complex; RGM: Rapidly growing mycobacteria (form colonies on subculture in less than 7 days); SGM: Slow growing mycobacteria (require 7 days or more to form colonies on subculture) [33].

1.3.2 Transmission

Disease in humans occur because the habitats occupied by NTM, including community and domestic drinking water distribution systems, are shared with humans. Although the modes of transmission of NTM remain unclear, the following mechanisms have been proposed as the likely routes of transmission from environmental reservoirs:

(1) Ingestion of soil or water by children leading to cervical lymphadenitis.

(2) Inhalation of NTM-laden aerosols by adults leading to pulmonary disease.

(3) Aspiration into the lungs by gastric reflux of ingested NTM from the stomach, leading to pulmonary NTM disease.

(4) Oral ingestion of NTM in water associated with reduction of the normal barrier function of the gastrointestinal tract, leading to pulmonary NTM disease in patients with profound immunodeficiency. For example, genotyping of isolates from some patients with pulmonary MAC matched them to MAC isolates obtained from their household water systems [18].

(5) Direct inoculation of NTM into skin resulting in ulcers. For example, *M. marinum*, a slow-growing mycobacterium, commonly causes a cutaneous disease otherwise known as swimming pool or fish tank granuloma in fish tank fanciers. However, use of swimming pool slides and contact with fish have all been associated with development of *M. marinum* disease, both in developed and developing countries [2].

Unlike its highly pathogenic relative MTBC, it is believed neither human-to-human nor zoonotic NTM disease occur. However, the likelihood of human-to-human spread has been raised by genotyping reports of identical *M. abscessus* ss. *massiliense* in an outbreak among

patients with cystic fibrosis (CF) seen in the same clinic [34]. Evidence in support of this is drawn from the extensive evaluations of the clinic that did not find any environmental source of the pathogen. In addition, these patients had no common environmental or other exposure beyond shared time within the CF centre. Therefore, research aimed at improving our understanding of NTM transmission should be a priority as this may provide a means of intervening to prevent disease in the susceptible host.

1.3.3 Risk factors for non-tuberculous mycobacteria diseases

Although NTM are widespread in the environment and humans are literally surrounded by these organisms, [35] relatively few people develop NTM disease, suggesting that disease is opportunistic and that some individuals have an intrinsic vulnerability.

Secondary immunodeficiency due to HIV/AIDS is the most common reported risk factor for NTM diseases. In persons living with HIV, disseminated NTM disease typically occur when they are severely immunosuppressed i.e. $CD4^+$ T-lymphocyte counts have fallen below 50/µl, suggesting that specific T-cell products or activities are required for NTM resistance [2, 36]

Similarly, evidence from patients with Mendelian susceptibility to mycobacterial disease, a primary immunosuppressive condition, highlights the importance of the Interleukin (IL) 12/23-Interferon (IFN) gamma pathway in preventing disseminated disease with NTM [4]. Abnormal α_1- antitrypsin (AAT) phenotypes have also been identified as risk factors for NTM idisease [37]. In addition, immunosuppressive chemotherapy for cancer and other autoimmune and rheumatoid conditions are the most common risk factors for *M. avium*, *M. intracellurare*, *M. abscessus* and *M. kansasii* diseases.

Lung damage resulting from previous TB disease, chronic obstructive pulmonary disease (COPD), occupational exposures to dusts (e.g., mining), CF, bronchiectasis, pneumoconiosis, pulmonary alveolar proteinosis, all of which reduce pathogen clearance in the lungs, increase susceptibility to NTM colonization and disease [2, 7, 38-40].

In the United States of America and Europe, pulmonary NTM disease has also been reported frequently in tall, slender, post-menopausal women without recognized predisposing factors [2, 7, 38]. This has led investigators to speculate that abnormal expression of sex hormones may play an important role in their susceptibility. For unknown reasons, women are more likely to have pulmonary NTM disease than men and pulmonary NTM disease prevalence appears to rise with increasing age (Figure 3) [41, 42]. The immunosenescence associated with advancing age is a well-described phenomenon associated with an increased susceptibility to disease, including mycobacteria.

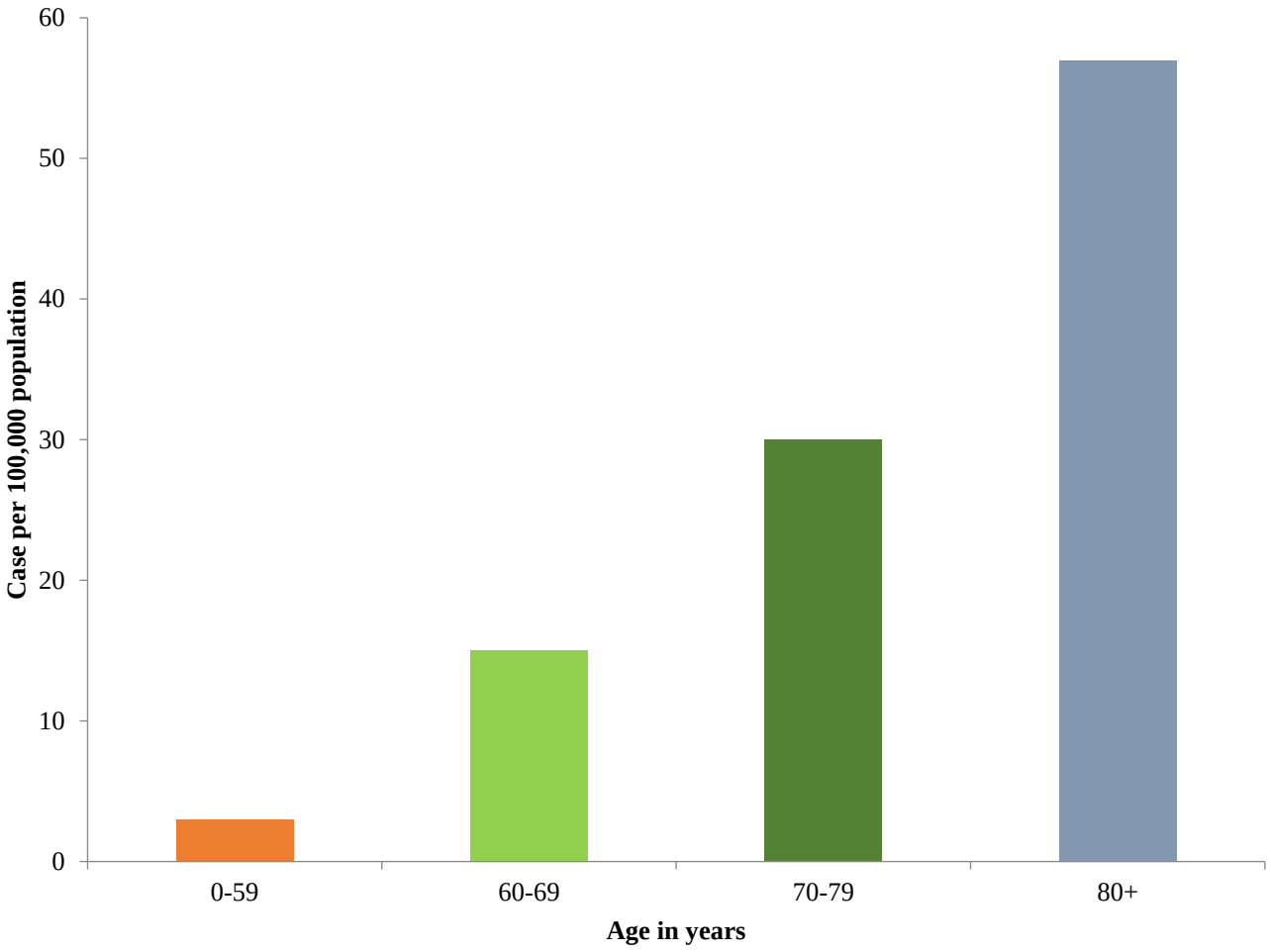

Figure 3: Average annual prevalence of pulmonary non-tuberculous mycobacteria disease by age at four intergrated health care delivery systems, (Group Health and Kaiser Permanente Southern California combined), 2004-2006 [42].

1.4 Human non-tuberculous mycobacteria colonisation and disease

1.4.1 Non-tuberculous mycobacteria disease

Given the pervasive nature of NTM in the environment, they are very commonly seen as commensals (temporary or permanent) of the human respiratory or digestive tract [13, 25, 37, 43, 44]. As a consequence of this, presence of antibodies to a common mycobacterial antigen, lipoarabinomannin (LAM), in settings where MTBC is uncommon, is predominantly

attributed to NTM colonisation [45, 46]. While colonisation rates of 1-15/100,000 persons have been reported in North America and Europe, [2, 5] the prevalence of NTM carriage is largely unknown for most countries in sub-Saharan Africa. There is value in investigating their prevalence in carriage and disease in this region of the world where MTBC is more common and NTM colonisation may have implications for diagnosis of MTBC, especially in pulmonary samples. In addition, it is possible that cases of NTM disease especially pulmonary manifestations may be masked by or missed because of the focus on MTBC as part of TB control efforts.

Skin test studies in adults using NTM sensitins (antigens) indicate that a substantial proportion have had prior clonisation with NTM [46, 47]. For instance, skin test studies with an *M. intracellulare* purified protein derivative (PPD-B) conducted among Navy recruits in the United States of America in the 1960s showed reactions of greater than four millilitres induration were more common in the south-eastern than northern United States, suggesting higher background rates of NTM colonisation in these areas [47]. Reactions to skin tests derived from NTM are not sufficiently species-specific to indicate which NTM might have been responsible for these asymptomatic colonisation, and it is possible that cross-reactivity with MTBC disease contributed to some of these reactions. However, because MAC organisms are the most common cause of NTM disease in the United States, it is likely that MAC was also the most common cause of colonisation. Compared to MTBC, NTM carriage is not like latent *M. tuberculosis* infection, as it is not associated with any potential for progression to, or reactivation of, active disease.

Differences in NTM species distribution have been proposed as a mechanism to explain the variable geographical efficacy of (0-80%) Bacille Calmette–Guerin (BCG) vaccination [48,

49] while other studies have shown that adaptive immunity developed by exposure to NTM in some regions of the world may provide cross-protection against TB and leprosy diseases [50]. Studies on subjects in the United Kingdom and Malawi found population differences in immune responses following BCG vaccination that were attributed to differential sensitisation due to exposure to NTM [51]. [49, 50].

1.4.2 Non-tuberculous mycobacteria disease

Like MTBC, NTM have the potential to infect and cause disease in any organ or part of the body given the opportunity, especially in the immunocompromised host. The spectrum of disease ranges from self-limited furunculosis to life-threatening disseminated diseases.

1.4.2.1 Pulmonary non-tuberculous mycobacteria disease and recent trends

The most common clinical manifestation of NTM is pulmonary disease [7, 52-56] and this makes up about 90% of all NTM diseases. Although there are more than 150 NTM species, pulmonary disease is most commonly due to MAC followed by *M. abscessus/M. chelonae* as shown in Figure 4. More recently, unusual NTM species associated with lung disease such as *M. arupense*, *M. cosmeticum*, *M. iranicum*, *M. kubicae*, *M. monascence*, *M. novocatrense*, *M. tusciae* and *M. yongonense* [57], *M. szulgai* [58], *M. porcinum* [59] and *M. massiliense* [60] have been described in the United States, Asia and Europe.

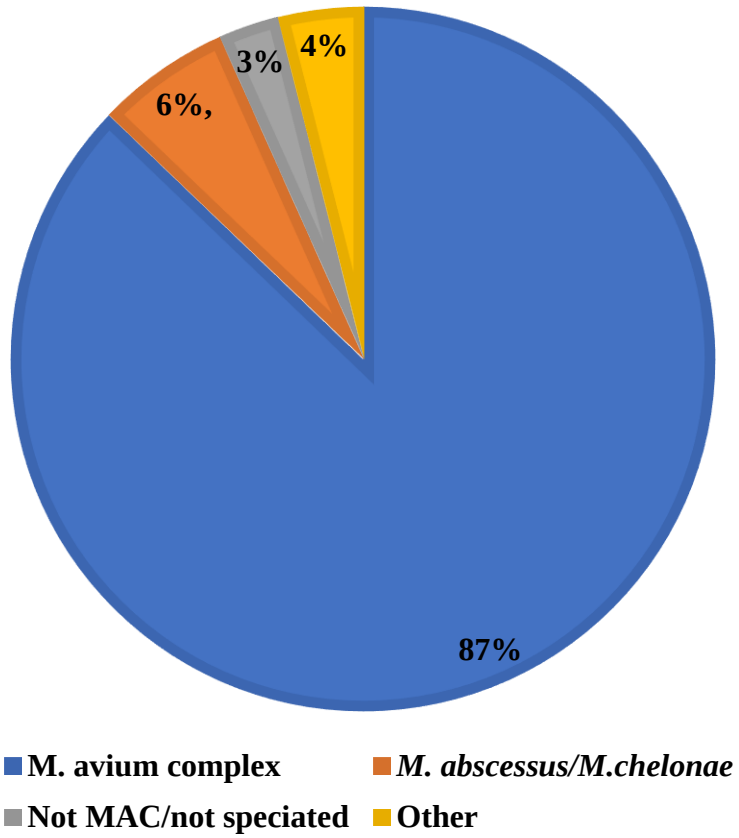

Figure 4: NTM species distribution in confirmed pulmonary non-tuberculous mycobacterial patients, Oregon 2005 to 2006 [61].

Over the last three decades, the incidence of NTM isolated from pulmonary samples and pulmonary NTM disease has shown an increasing trend worldwide. In the United States, the prevalence of pulmonary NTM disease more than doubled between 1997 and 2007 while TB disease incidence remained stable or declined [41]. Similarly, Koh et al. reported increasing recovery of NTM from respiratory specimens in a tertiary referral hospital in South Korea, as shown in Figure 5 [62].

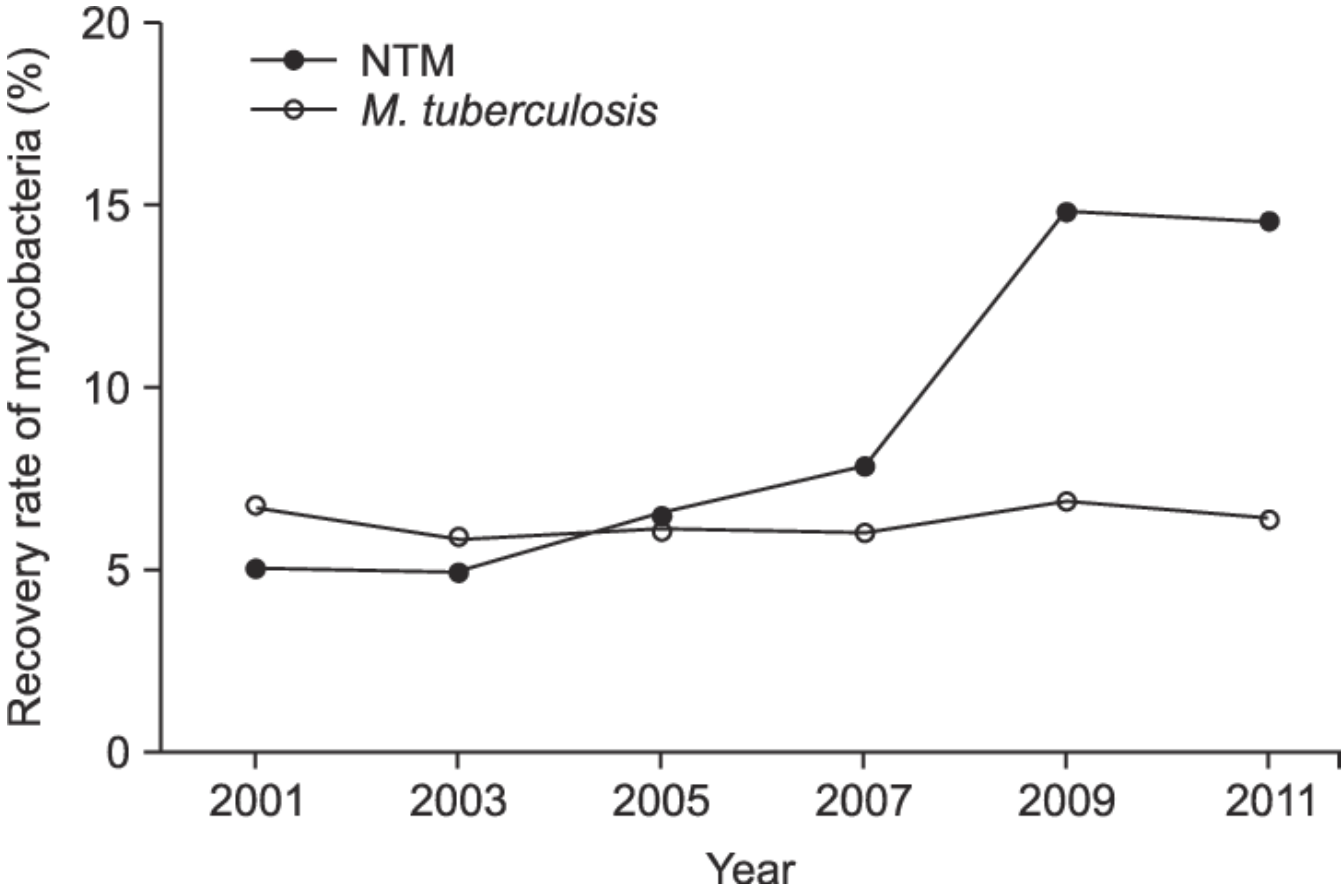

Figure 5: Increasing recovery of non-tuberculous mycobacteria from respiratory specimens over a 10-year period in a tertiary referral hospital in South Korea [63].

Other investigators in Croatia also found MTBC isolates from pulmonary samples declined two-fold while the number of NTM isolates almost doubled in the same period (Figure 6) [27].

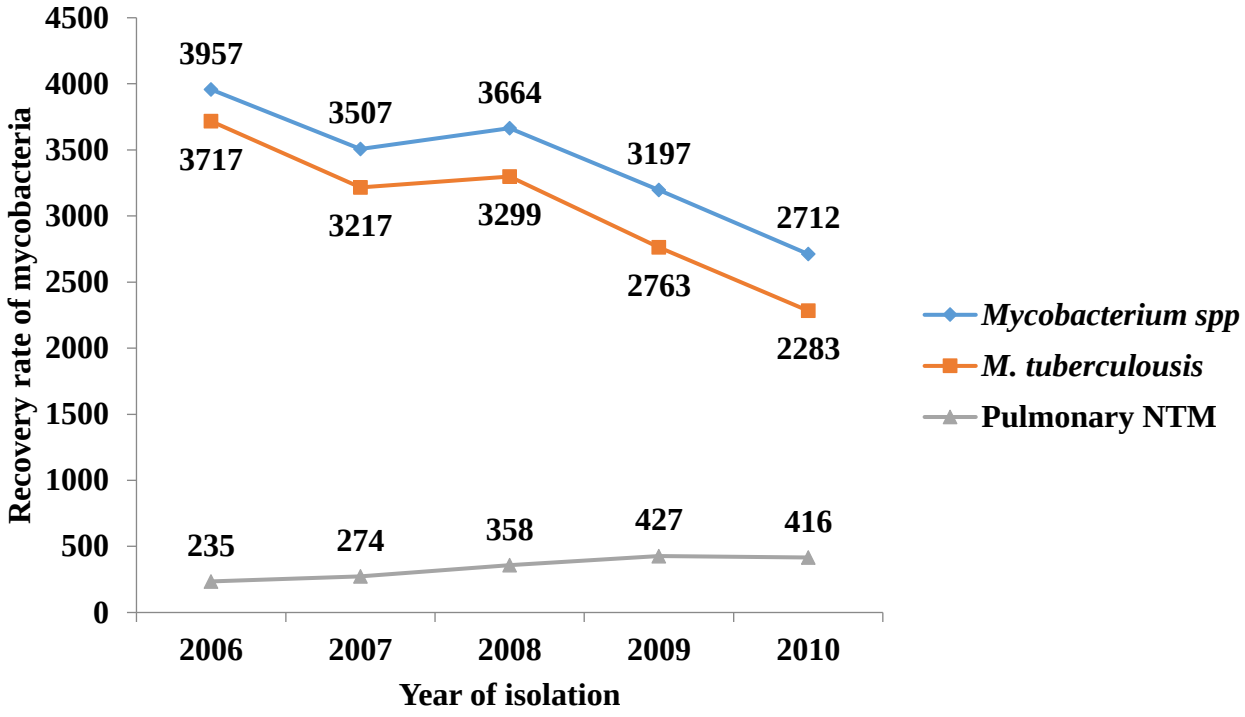

Figure 6: Total number of *Mycobacterium* spp., *M. tuberculosis* and pulmonary NTM isolates submitted to the Croatian national reference laboratory during the study period 2006-2010 [27].

More recently, Yoon and colleagues showed that the age-adjusted prevalence of pulmonary NTM colonisation in women was higher than that in men, and the differences increased by year; the prevalence rates for men and women between 2009 and 2016 were 8.6 and 25.3, and 10.3 and 46.8, respectively. However, the prevalence and incidence of TB per 100,000 population decreased between 2009 and 2016 (106.5 and 74.4 for prevalence; 81.2 and 61.8 for incidence) as illustrated in Figure 7 [64].

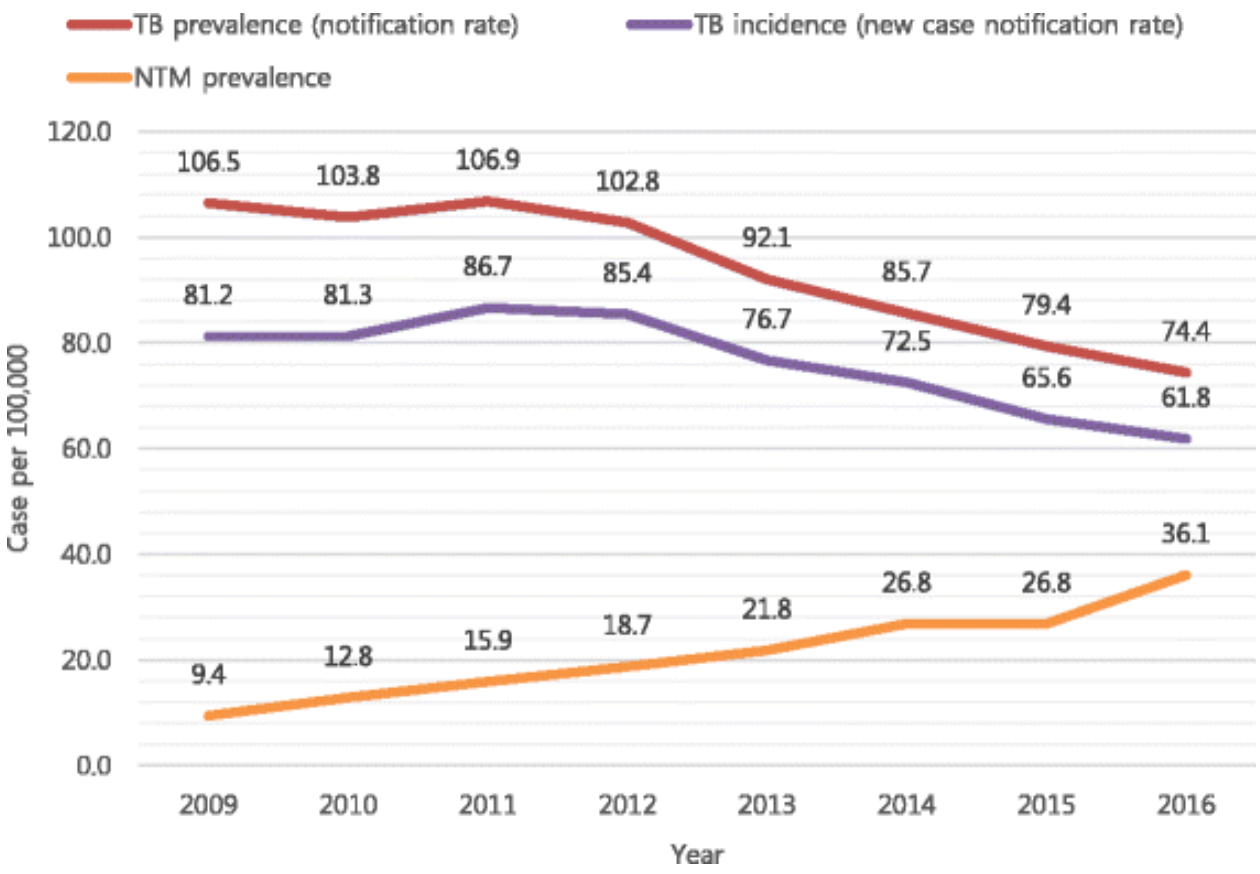

Figure 7: Prevalence and incidence of tuberculosis, and prevalence of nontuberuclous mycobacterial diseases per 100,000 population in Korea (2009-2016) [64].

This relative increase in NTM isolation and pulmonary NTM disease has occurred while TB cases declined leading to speculation that waning cross-protective mycobacterial immunity from TB disease might have contributed to an increase in NTM disease [2, 65]. Although there might be a true increase in disease prevalence, alternative explanations for this observation include declining MTBC disease, improved laboratory techniques [1, 2], greater disease awareness [7], pseudo infections from hospital contamination (during collection of specimen), and /or laboratory contaminations (during specimen processing) [7, 66, 67].

1.4.2.2 Challenges with describing the global epidemiology of pulmonary non-tuberculous mycobacterial disease

Most of the available data on NTM lung disease prevalence is reported from United States, middle-income Asian countries, and Europe [2, 7, 68]. There are several obstacles in the way of obtaining detailed pulmonary NTM disease epidemiology for other parts of the world. First, as NTM diseases are not notifiable, there are no formal surveillance systems in place to capture data and case data are not usually reported to Public Health Authorities in many regions of the world, especially in sub-Saharan Africa. Secondly, the overlapping clinical manifestations with MTBC disease in high TB burden settings make the specific diagnosis of NTM disease difficult. For example, slow growing NTM such as *M. kansasii* and *M. szulgai* cause pulmonary tuberculous-like disease making the differential diagnosis of pulmonary NTM and TB disease challenging [1, 58]. Finally, NTM can colonise the respiratory tract, finding NTM in respiratory secretions does not necessarily confirm clinical disease [2, 5, 69].

In 1997, the American Thoracic Society and Infectious Diseases Society of America (ATS/IDSA) developed a rigorous case definition for differentiation of casual NTM isolation from true pulmonary NTM disease. These guidelines were revised in 2007 to include more lenient diagnostic criteria and require clinical, radiological and microbiological evidence for diagnosis (see Table 2) [2]. Unfortunately, in most epidemiological studies, the difficulty of obtaining complete radiographic and clinical data has prevented accurate assessment of true pulmonary NTM disease prevalence [7, 37]

Table 2: Summary of the American Thoracic Society/Infectious Disease Society of America Diagnostic criteria for pulmonary non-tuberculous mycobacterial disease

Clinical
1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or a high-resolution computed tomographic scan that shows multifocal bronchiectasis with multiple small nodules.
And
2. Appropriate exclusion of other diagnoses.
Microbiologic
1. Positive culture results from at least two separate expectorated sputum samples (If the results from the initial sputum samples are non-diagnostic, consider repeat sputum acid-fast bacillus (AFB) smears and cultures).
OR
2. Positive culture results from at least one bronchial wash or lavage.
OR
3. Transbronchial or other lung biopsy with mycobacterial histopathological features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathological features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.
4. Expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination
5. Patients who are suspected of having NTM lung disease but who do not meet the diagnostic criteria should be followed until the diagnosis is firmly established or excluded.

1.5 Non-tuberculous mycobacteria extra pulmonary disease

Approximately 10% of all NTM disease manifest as extra pulmonary disease [55, 70]. The portals of entry are the respiratory tract, gastrointestinal tract, or direct inoculation through trauma or invasive procedures. Common extra pulmonary disease manifestations include cervical lymphadenitis, disseminated disease, skin, and soft tissue and bone diseases. Keratitis, catheter-related bloodstream diseases, septic arthritis and central nervous system diseases also occur but are uncommon.

The rapid growers, including *M. chelonae*, *M. abscessus*, and *M. fortuitum* are predominant causative agents [70]. The incidence and prevalence of extra pulmonary disease is unknown worldwide. Where outbreaks have been reported, these are mainly due to inadequate disinfection of surgical equipment or contamination of injected solutions or medications [2, 16].

1.6 Treatment of non-tuberculous mycobacterial diseases

NTM are relatively resistant to antimicrobial compounds owing to their impermeable cell wall and broad repertoire of efflux pumps characteristic of mycobacteria [71]. Nonetheless, the availability of newer macrolides/azilides have drastically improved this situation and are now the cornerstones of therapy for most NTM diseases [2]. Azithromycin and clarithromycin have excellent *in vitro* activity against MAC [72] and are able to achieve penetration into phagocytes and tissues [73]. These drugs have therefore become central to the treatment of MAC lung diseases and extrapulmonary diseases. For *M. kansasii* disease, prolonged triple drug therapy including isoniazid (INH), rifampin, and ethambutol is recommended. Patients are treated until sputum culture negative on therapy for at least 12

months. Recently, macrolides such as clarithromycin and the fourth-generation fluoroquinolone, moxifloxacin, have been shown to have *in vitro* activity against this organism and may soon become an alternative to INH [74].

Diseases caused by rapidly growing mycobacteria, especially *M. abscessus,* are notoriously difficult to treat successfully with drug therapy alone. Chemotherapy in conjunction with surgical resection is often needed in those who can tolerate it and IFN gamma has been used in some trials [70].

Studies of phylogeny, drug susceptibility, and clinical significance of mycobacteria have led investigators to hypothesize that drug susceptibility in mycobacteria is acquired and reflects the level of competition in, and adaptation to, the human (environmental) niche of the mycobacteria. This is supported by the fact that the most virulent NTM (*M. marinum, M. ulcerans, M. kansasii, M. szulgai,* and *M. malmoense*) are most adapted to humans and phylogenetically closer to MTBC compared to other NTM. In addition, they possess the ESX-1 system (a type VII secretion system responsible for the secretion of Early Secreted Antigenic Target 6 (ESAT-6) and Culture Filtrate Protein (CFP) 10 proteins, which are crucial for *M. tuberculosis* complex virulence), are characterized by high levels of natural drug susceptibility and cause disease that are most treatable [2, 22, 75, 76]. In contrast, NTM that inhabit the more competitive environmental niches such as *M. gordonae*, *M. fortuitum*, *M. chelonae,* and *M. simiae* are phylogenetically distant to MTBC, and have a low clinical significance because they are the least adapted to humans. When found in disease, curative therapy is difficult because they are most tolerant to antibiotics derived from microbes with which they share their habitat (Figure 8).

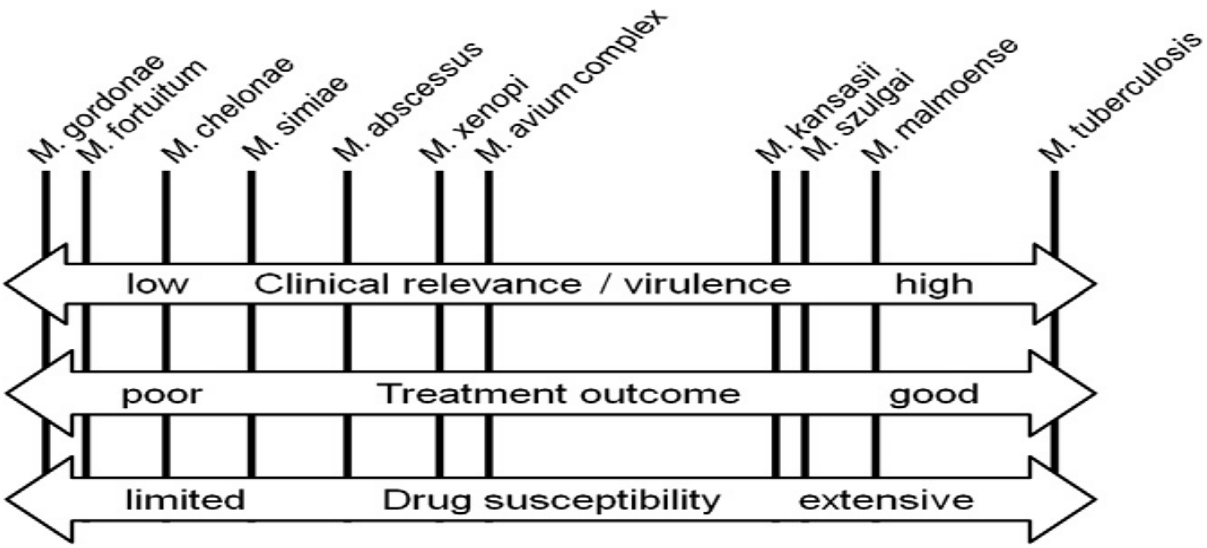

Figure 8: Relationships between virulence, treatment outcome and drug susceptibility visualized. Note: Clinical relevance and virulence, as well as treatment outcome herein are based on pulmonary disease [75].

1.7 Laboratory diagnosis of pulmonary non-tuberculous mycobacteria disease

1.7.1 Smear microscopy and sample decontamination

NTM are acid and alcohol fast bacilli (AAFB) and can be detected by the classical Ziehl-Neelsen (ZN) and fluorochome staining techniques just like for MTBC [77]. As with MTBC, smear microscopy has poor sensitivity for diagnosing NTM diseases when the bacterial load is less than 10,000 organisms/ml in sputum sample Ref. The sensitivity of microscopy has however, increased considerably with improved techniques and standardization of sputum preparation. These include:

(1) Liquefaction with N-acetyl-L-cysteine (NALC) and 2% sodium hydroxide (NaOH)

(2) Concentration of the sputum by centrifugation.

(3) Promotion of the use of auramine-rhodamine with the fluorochrome method instead of the classic acid-fast stains of ZN and Kinyoun, which use carbol-fuchsin [1, 2].

Specimens from non-sterile sites, such as sputum, require decontamination to avoid overgrowth by fast growing bacteria or fungi. Decontamination with final concentrations of 1% NaOH is most commonly used. An increase of NaOH concentrations from 1% to 1.25% has been shown to lower contamination rates but also leads to a 10% decrease in detection of NTM [78]. Buijtels *et al.* demonstrated the utility of sulphuric acid (final concentrations of 3%) in improving detection rates of NTM compared with 1% NALC-NaOH, by liquid culture, without an increase in contamination rates [79].

1.7.2 Culture conditions and media

Liquid and solid media cultures are recommended for recovery and detection of NTM [2]. As with MTBC, liquid media culture systems such as the Mycobacteria Growth Indicator Tube (MGIT) methods in general are more sensitive than solid media such as Lowenstein-Jensen, Ogawa, and Middle brook 7H10/7H11 [80-82]. In addition, these media offer the advantage of more rapid growth, thus significantly reducing delays to diagnosis and treatment. However, liquid culture media are more susceptible to bacterial overgrowth and contamination. Solid media cultures allow for visualization of bacterial colony characteristics and morphology, and serve as a backup when liquid cultures become contaminated [83].

Typical of environmental organisms, NTM are versatile in their metabolic needs. Yet, for a small number of species, *M. genavense* and *M. haemophilium* for example, enrichment of culture media is needed to facilitate growth. In the case of *M. tilburgii*, attempts to isolate it

in culture have not been successful despite its presence in large numbers in clinical samples [84].

The optimal temperature for NTM cultures is between 25°C and 37°C. The culture conditions for NTM are shown in Table 3.

Table 3: Culture conditions for primary isolation of non-tuberculous mycobacteria species

NTM species	Optimal growth temperatures	Special requirements for growth
M. avium complex *M. kansasii, M. malmoense* *M.abscessus, M. chelonae* *M. fortuitum, M. gastri* *M.gordonae*	32°C to 42°C	Not applicable (N/A)
M. genavense	37°C	Human blood, charcoal, casein, and yeast extracts, acidified to pH 6, mycobactin J
M. conspicuum	22°C to 30°C	N/A
M. haemophilum	28°C to 32°C	Iron-containing compounds i.e. ferric ammonium citrate, hemin, or hemoglobin
M. marinum	25°C to 32°C	N/A
M. ulcerans	30°C	N/A
M. lentiflavum	22°C to 37°C	N/A
M. thermoresistible	37°C to 52°C	N/A
M. smegmatis *M. xenopi*	37°C to 45°C	N/A

1.7.3 Laboratory Identification of non-tuberculous mycobacteria

Species-level identification of the NTM is becoming increasingly clinically important because of differences in antimicrobial susceptibility that underpin treatment options. NTM species differ in their ability to cause human pulmonary disease and the clinical relevance of NTM species have geographic variations [37, 85].

Some other organisms belonging to the genus *Nocardia/Rhodococcus* have the same acid and alcohol fast appearance as NTM and MTBC on microscopy. Pulmonary nocardiosis is often misdiagnosed and treated as TB, sometimes with fatal consequences. Therefore, accurate identification of NTM by molecular techniques and utility of *in vitro* drug susceptibility testing for different NTM species are critical for accuracy and for effective treatment for pulmonary NTM diseases [2, 13, 28, 86].

The methods for identification of NTM in clinical laboratories have evolved over the past two decades from culture-based, phenotypic and biochemical identification to molecular techniques. These molecular diagnostic approaches are less cumbersome than conventional microbiological techniques with reduced turnaround time and significantly increased sensitivity, higher discriminative power and reproducibility [1, 22, 87].

Among molecular methods, two approaches are commonly used. The first are line probe assays which are easy to perform and allow a reasonable level of discrimination and identification of the most frequently encountered species such as MAC, *M. kansasii, M. abscessus* and *M. gordonae.* Second is partial gene sequencing which allows a higher level of discrimination, often up to subspecies level, but is only feasible for laboratories with access to sequencing facilities. A well documented example is the *16S rRNA* gene sequencing

technique. The *16S rRNA* gene is the first and the best established technique for bacterial typing and was widely used before the other molecular techniques. It is ~1,500 nucleotide-sequence encoded by the 16S ribosomal deoxyribonucleic acid (rDNA). This gene is present in all bacteria, containing highly conserved regions flanked by hyper variable regions making it an important target for bacterial identification [88]. The bacterial *16S rRNA* gene contains nine hypervariable regions (V1-V9) ranging from about 30-100 base pairs long. The degree of conservation varies widely between hypervariable regions, with more conserved regions correlating to higher-level taxonomy and less conserved regions to lower levels, such as genus and species. Tortoli *et al*. have shown that the most interesting nucleotide for mycobacteria *16S rRNA* gene investigations are those shared by all the members of the genus mycobacteria and also the hypervariable regions characterised by species specific variability [22]. The *16S rRNA* -based genetic investigation of mycobacterial phylogeny and taxonomy focusses on two hypervariable sequences, known as region A (V3) and B (V5), that correspond to the *Escherichia coli* positions around 130 to 210 and 430 to 500 respectively (Figure 9)The *16S rRNA* gene sequencing analysis was the method of choice for identification of NTM in this study.

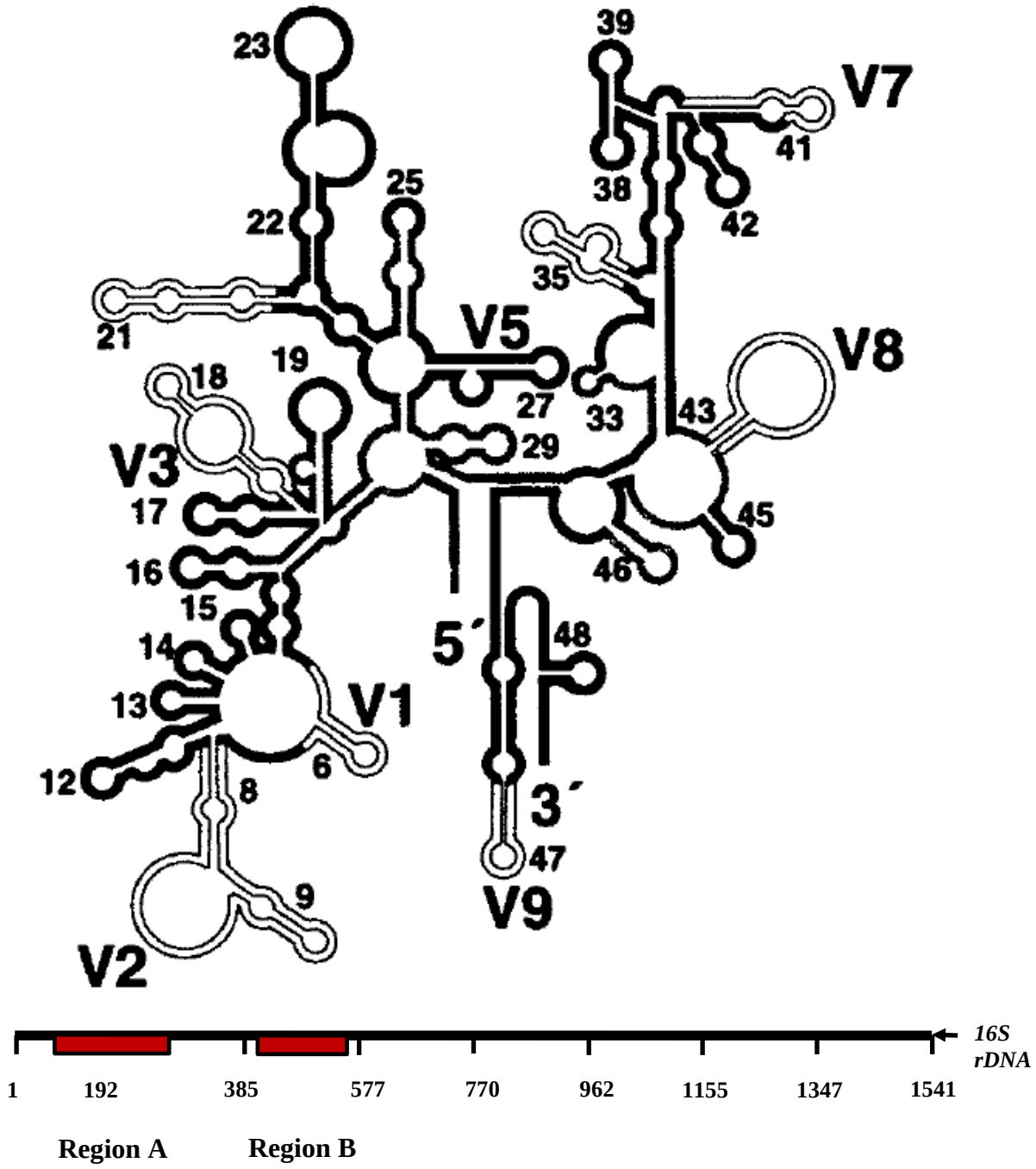

Figure 9: Diagramatic representation of *16S rRNA* gene highlighting region A(V3) and B(V5) for *16S rRNA*-based genetic investigations of mycobacteria phylogeny and taxonomy. Regions A and B correspond to *Escherichia coli* positions 130 to 210 and 430 to 500 respectively. Figures (1, 192 etc.) represent base pairs.

Recently, the matrix assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) has been developed for NTM species identification[89]. This is an ionization technique that uses a laser energy absorbing matrix to create ions from large molecules with minimal fragmentation. It has been applied to the analysis of biomolecules and large organic molecules which tend to be fragile and fragment when ionized by more conventional ionization methods. MALDI-TOF mass spectrometry has revolutionized speed and precision of microbial identification for clinical isolates outperforming conventional methods. Recent studies on the utility of the MALDI-TOF technique for NTM identification showed that for rare NTM species, identification is sometimes not possible, but in most cases, the results of identification by the MALDI-TOF MS were concordant with the results obtained from conventional real time PCR and DNA hybridization methods.[89, 90] However, the optimal method for protein extraction from mycobacteria and the true discriminatory power of this method are yet to be established.

2 Rationale for Thesis

Data on the contribution of NTM to pulmonary disease in sub-Saharan Africa is uncertain. As highlighted in chapter 1, sub-Saharan Africa was poorly represented in the 'global' NTM collection that led to a description of the global epidemiology of NTM in colonisation and disease [13]. The scarcity of data on the contribution of NTM to pulmonary mycobacterial infections in sub-Saharan Africa is most likely due to the following reasons:

(1) Poor awareness among treating physicians and microbiologists on the clinical relevance of NTM.
(2) Lack of laboratory infrastructure for culture and identification of mycobacterial isolates.
(3) The high burden of TB and HIV in this region of the world attracts the bulk of the attention of the health care system. Therefore, the fiscal inputs required for diagnosis and treatment NTM disease are lacking.
(4) Misdiagnosis - since sputum smear microscopy is the mainstay of TB diagnosis in most high burden TB settings, the risk of acid-fast NTM in pulmonary samples, especially in previously treated patients with lung damage, poses a diagnostic challenge.

Given the paucity of data on NTM and the little understanding of the epidemiology of pulmonary NTM in disease and colonisation in the sub-Saharan Africa, I conducted a comprehensive, systematic review of pulmonary NTM colonisation and disease in the region, and investigated the epidemiology and population biology of NTM found in pulmonary samples in a nationwide survey in The Gambia.

2.1 Study hypothesis

Non-tuberculous mycobacteria are prevalent in sputum samples of TB suspects in sub-Saharan Africa and can cause pulmonary TB-like disease

2.2 Aims of study

- To consolidate existing data on pulmonary NTM colonisation and disease (according to the ATS/IDSA criteria) in sub-Saharan Africa, review the existing gaps in our knowledge of pulmonary NTM and identify future research priorities.
- To estimate the prevalence and predictors of NTM colonisation and disease in pulmonary samples of a representative subset of the Gambia population

3 Non-tuberculous Mycobacteria Isolated from Pulmonary Samples in sub-Saharan Africa – A Systematic Review of the Literature.

Catherine Okoi, Suzanne T. Anderson, Martin Antonio, Sarah N. Mulwa, Florian Gehre, Ifedayo M.O. Adetifa

(Submitted to Scientific Reports 7th February 2017; Revised manuscript re-submitted 24th June 2017)

3.1 Abstract

Background

Pulmonary NTM disease epidemiology in sub-Saharan Africa is not as well described as for pulmonary tuberculosis. Earlier reviews of global pulmonary NTM epidemiology only included subjet-level data from one sub-Saharan African country. With the increasing incidence of case reports and series from diverse countries and regions of the world, the distribution of NTM species isolated from pulmonary samples appear to vary significantly by region. However, very little is known about the contribution(s) of NTM to tuberculosis-like disease, and significant knowledge gaps exist regarding their geographical distribution, clinical and molecular epidemiology in low and middle income countries where there is a high burden of disease caused by MTBC.

Methods

We systematically reviewed the literature and searched electronic data bases (PubMed, Embase, Popline, OVID and Africa Wide Information) for articles on prevalence and clinical relevance of NTM detected in pulmonary samples in sub-Saharan Africa. We applied the American Thoracic Society/Infectious Disease Society of America criteria to differentiate between colonisation and disease.

Findings

Only 37 articles from 373 citations met our inclusion criteria. The prevalence of pulmonary NTM colonisation was 7.5% (95% CI: 7.2% - 7.8%), and 75.0% (2325 of 3096) occurred in males, 16.5% (512 of 3096) in those previously treated for tuberculosis and *Mycobacterium avium* complex predominated (27.7% [95% CI: 27.2 – 28.9%]). In seven eligible studies, 27.9% (266 of 952) of participants had pulmonary NTM disease and *M. kansasii* with a prevalence of 69.2% [95% CI: 63.2 – 74.7%] was the most common cause of pulmonary NTM disease. NTM species were unidentifiable in 29.2% [2,623 of 8,980] of isolates.

Interpretation /Significance

In conclusion, pulmonary NTM disease is a neglected and emerging public health disease in sub-Sharan Africa that requires enhanced surveillance to fully quantify the diversity of and contribution of NTM to pulmonary disease and to reduce the risk of misdiagnosis of tuberculosis.

3.2 Introduction

The epidemiology of pulmonary disease caused by *Mycobacterium tuberculosis* complex (MTBC) - *M. tuberculosis, M. bovis, M. africanum, M. canetti, M. microti, M. pinnipedii* and *M. caprae* - is better known than for NTM [7]. NTM is a designation used for a large number of potentially pathogenic and non-pathogenic environmental mycobacterial species other than MTBC and *Mycobacterium leprae*.

Worldwide, pulmonary disease caused by NTM are gaining increased attention, in part, because of increasing recognition and isolation in clinical settings and there are some better known NTM pathogens like *M. avium subsp paratuberculosis* and *M. marinum*

[55, 56]. Although NTM were identified soon after Koch's identification of *M. tuberculosis* as a cause of active tuberculosis in 1882, it was not until the 1950s that NTM were recognized to cause human pulmonary disease. Given their ubiquitous presence in the environment, it is important to distinguish colonisation from active disease following isolation of NTM from pulmonary samples. In response to this challenge, the ATS/IDSA introduced stringent diagnostic criteria with clinical, radiological and microbiological components for diagnosis of pulmonary NTM disease [2].

The clinical and molecular epidemiology of prevalent NTM in low and middle-income countries also endemic for pulmonary tuberculosis is less known because pulmonary and other disease manifestations caused by NTM pose a diagnostic challenge to microbiologists and clinicians [1, 2]. In contrast to pulmonary tuberculosis, it is not possible to readily identify pulmonary NTM disease with the usual combination of basic mycobacteriology, clinical history, radiologic imaging and the tuberculin skin test, where applicable. The culture and molecular biology identification techniques required for NTM diagnosis are not cost

effective for routine clinical practice in resource-poor health systems where priority is currently given to expanding access to diagnosis and treatment for pulmonary tuberculosis [10, 53]. Distribution of NTM species isolated from pulmonary samples differs significantly by geographic region. However, most of these data are from the developed world and sub-Saharan Africa is under represented [13, 33]. Although there are now emerging NTM disease data from Asia and parts of Africa, significant knowledge gaps still exist especially in sub-Saharan Africa where nine of the world's 22 high burden tuberculosis countries are found

[11-13]. Therefore, fears that inconclusive diagnosis based on smear microscopy or clinical symptoms and/or radiological findings could lead to misdiagnosis of pulmonary tuberculosis and/or inappropriate management of pulmonary NTM cases are valid. The objectives of this review were to consolidate existing data on pulmonary NTM in sub-Saharan Africa, review the existing gaps in our knowledge of pulmonary NTM and identify future research priorities.

3.3 Methods

3.3.1 Literature Search and Selection Criteria

This review was conducted in accordance with PRISMA guidelines [91]. The overall aim of this review was to determine the prevalence of NTM in apparently healthy and sick persons in sub-Saharan Africa. We defined sub-Saharan Africa as all of Africa except Northern Africa.

We searched PubMed, EMBASE, POPLINE, OVID and Africa Wide Information electronic databases for publications about pulmonary NTM in sub-Saharan Africa published from January 1, 1940 to October 1, 2016 using the following search terms and strategy:

((((((("nontuberculous mycobacteria"[MeSH Terms] AND "africa south of the sahara"[MeSH Terms]) OR "mycobacterium infections, nontuberculous"[MeSH Terms]) AND "africa south of the sahara"[MeSH Terms]) OR "mycobacterium infections, nontuberculous"[MeSH Terms]) AND "africa south of the sahara"[MeSH Terms]) OR (("lung"[MeSH Terms] OR "lung"[All Fields] OR "pulmonary"[All Fields]) AND "nontuberculous mycobacteria"[MeSH Terms])) AND "africa south of the sahara"[MeSH Terms] AND (("1940/01/01"[PDAT]: "2016/10/01"[PDAT]) AND "humans"[MeSH Terms]).

3.3.2 Selection process and data abstraction

We found 373 citations from our database searches (see Figure 10). The titles and abstracts of all the articles were screened and full-text copies of those deemed relevant obtained. In addition, the reference sections of all the retrieved articles were screened to identify other eligible citations. Only articles reporting on pulmonary samples were included. For all relevant articles, we extracted the following data using a data extraction sheet: research setting, study period, population tested and numbers, NTM species isolated, method for NTM identification, prevalence of pulmonary NTM isolation/disease, HIV co-infection rate and risk factor(s) for NTM acquisition.

3.3.3 Data analysis

In estimating country-level and overall prevalence of NTM in sub-Saharan Africa, a pooled estimate was computed based on a simple meta-analysis of the reported prevalences. Each study was weighted according to its sample size and the exact binomial used to derive the 95% confidence intervals (95% CI). We checked all retrieved articles for application of the

ATS/IDSA diagnostic criteria (Table 4) for clinically relevant pulmonary NTM and recorded the proportion of patients meeting these criteria and NTM species responsible.

All extracted data were stored in Microsoft® Excel® (Microsoft Corporation, Redmond, Washington, United States) and analysis carried out in STATA™ version 12.1 (College Station, Texas, United States)

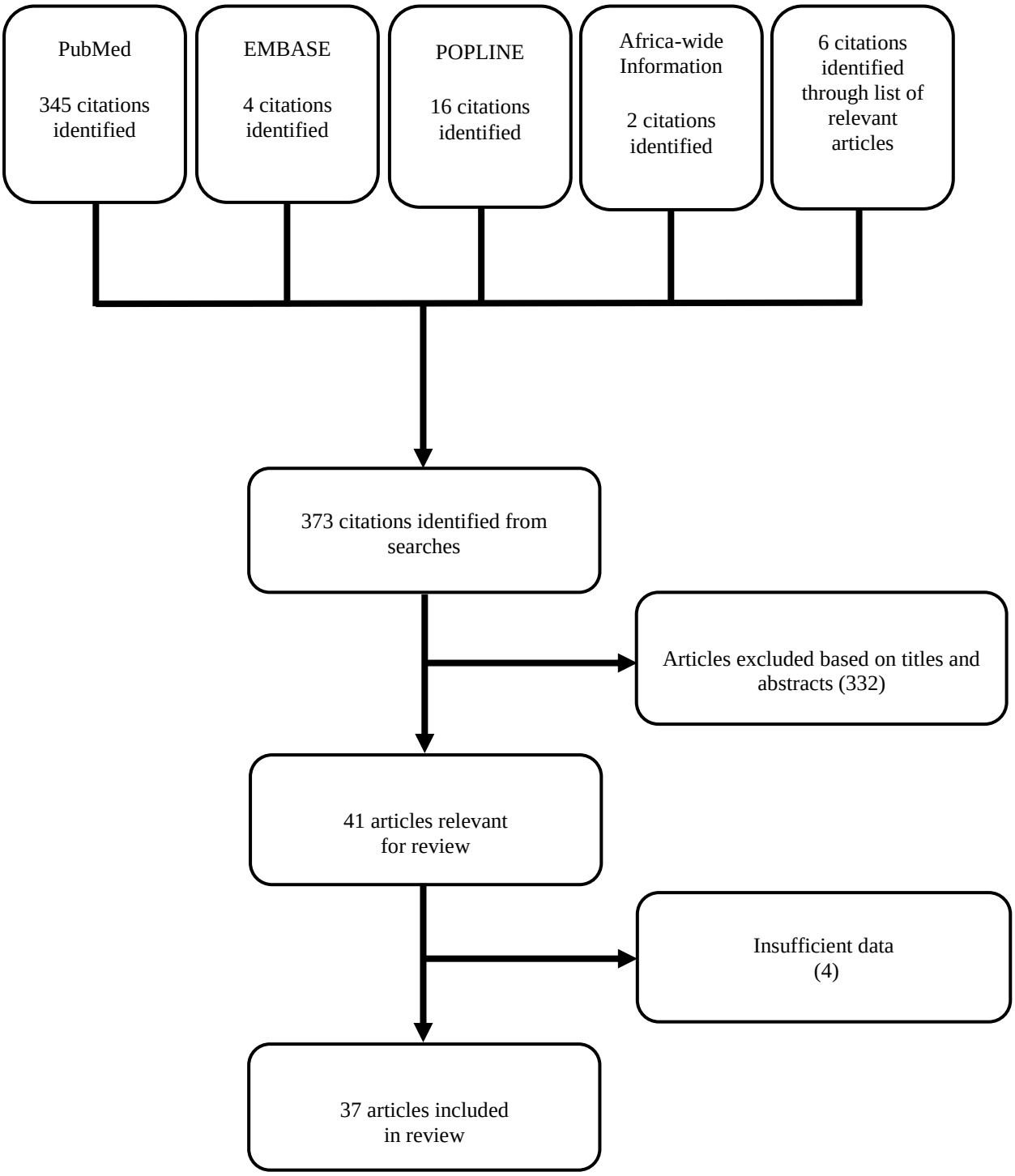

Figure 10: Flow chat on literature selection criteria and article search

3.4 Results

3.4.1 Distribution of included studies

There were only 37 relevant articles on the epidemiology of pulmonary NTM in sub-Saharan Africa as shown in Table 4. These were from studies in western (Nigeria, Mali and Ghana), southern (Zambia and South Africa [RSA]) and eastern (Kenya, Uganda, Tanzania and Ethiopia) Africa [9, 10, 13, 29, 38, 53, 69, 92-121]. Eleven articles were from Nigeria [53, 92-101] one from Mali [102] one from Ghana [103] six from Zambia [10, 79, 104-107] two from Kenya [108, 109] two from Uganda [110, 111] three from Tanzania [9, 112, 113] three from Ethiopia [114-116] and eight from South Africa [13, 29, 38, 117-121]. Where methods of identification were reported, molecular techniques (n= 26) were the most frequently used to identify NTM species, followed by conventional biochemical testing identification tools (n= 9) and immunochromatographic assays (n=2). The molecular diagnostic methods used were Restriction Fragment Polymerase Chain Reaction (RFPCR) of the 65KD *hsp* gene, Genotype CM/AS assay (Hain Life science, Nehren, Germany), and *16S rRNA* gene sequencing analysis in one, eleven and fourteen studies respectively. Identification methods also varied over time and a dramatic rise in the use of molecular methods was observed in the period 2000-2016. Biochemical and phenotypic tools were the only methods used for NTM identification before 2000. Despite this transition in identification methods used over time, there was no major change in diversity of NTM species isolated in the period before and after the year 2000 as shown in Table 5.

Table 4: Overview of studies on pulmonary non-tuberculous mycobacteria in sub Saharan Africa

Country	Study period	Reference	Age in years	Sample size	Sputum cultures		Most isolated NTM	Method of NTM identification	Overall prevalence of NTM isolation (%)	Pulmonary NTM patients with HIV coinfection (%)	ATS/IDSA applied/numbers meeting criteria	Risk factors for pulmonary NTM
					MTBC	NTM						
Ethiopia	2010	Mathewos et al. (2015) [115]	NA	263 presumptive TB cases	110	7	NTM not classified	Immunochro-matography assay (Capilia TAUNS method)	2.7	NA	No	NA
Ethiopia	2011	Workalemahu et al. (2015) [116]	1-15	121 presumptive TB cases	15	10	*M. fortuitum* *M. parascrofulaceum* *M. triviale*	Molecular (Sequencing of *16S rRNA* gene)	8.3	NA	NA	NA
Ethiopia	2008-09	Gumi et al. (2012) [114]	NA	260 presumptive TB cases	157	7	*M. flavescens*	Molecular (Sequencing of *16S rRNA* gene)	2.7	NA	No	NA
Ghana	2013-14	Bjerrum et al. (2016) [103]	≥18	473 HIV infected adults	60	38	*M. avium* complex *M. chelonae* *M. simiae* *M. fortuitum*	Molecular (sequencing of *16S rRNA* gene)	8.0	All HIV infected	No	HIV infection and age

Kenya	2007-09	Nyamogoba et al. (2012) [108]	≥0	872 presumptive TB cases	346	15	*M. fortuitum* *M. peregrinum*	Molecular (Genotype CM/As assay)	1.7	46.7	No	Previous TB HIV infection
Kenya	2014-15	Limo et al. (2014) [109]	≥0	210 retreatment cases	121	89	*M. intracellulare* *M. abscessus* *M. fortuitum*	Molecular (Genotype CM/As assay)	42.4	25.8	No	Previous TB infection
Mali	2004-09	Miaga et al. (2012) [102]	18-73	142 presumptive TB cases enrolled	113	17	*M. avium* *M. palustre* *M. fortuitum*	Molecular (sequencing of *16S rRNA* gene)	12.0	17.6	Yes; 11	Previous TB
Nigeria	2010-11	Olutayo et al. (2016) [92]		319 presumptive TB cases	122	26	NA	Molecular (Genotype CM/AS assay)	8.2	46.2	No	HIV infection, age
Nigeria	2008-09	Cadmus et al. (2016) [93]	NA	23 presumptive cases	11	9	*M. avium* complex	Molecular (Sequencing of *16S rRNA* gene)	39.1	NA	No	NA
Nigeria	2010-11	Gambo et al. (2014) [94]	NA	952 presumptive TB cases	254	65	NTM not classified	Molecular (Genotype CM/AS assay)	6.8	40.0	No	HIV infection, TB
Nigeria	2010-11	Gambo et al. (2013) [53]	18	1603 TB presumptive TB cases	375	69	*M. intracellulare* *M. abscessus* *M. fortuitum* *M. gordonae*	Molecular (Genotype CM/AS assay)	4.3	40.0	No	HIV infection, TB

Nigeria	2008-09	Asuquo et al. (2012) [95]	10-70	137 presumptive TB cases	81	4	*M. fortuitum* *M. avium species* *M. abscessus*	Molecular (Genotype CM/AS assay)	2.9	50.0	No	HIV infection
Nigeria	1983	Idigbe et al. (1986) [96]	NA	668 presumptive TB cases	NA	NA	*M. avium* *M. kansasii* *M. fortuitum*	Conventional biochemical methods	11.0	NA	NA	NA
Nigeria	1982-93	Idigbe et al. (1995) [97]	NA	NA	NA	NA	*M. avium* *M. kansasii* *M. xenopi* *M. fortuitum*	Conventional biochemical methods	NA	NA	No	NA
Nigeria	NA	Mawak et al. (2006) [98]	≥14	329 presumptive cases	50	15	*M. avium* *M. kansasii* *M. fortuitum*	Conventional biochemical methods	4.6	NA	No	NA
Nigeria	2007-09	Daniel et al. (2011) [99]	25-80	102 TB patients (41 new s+ and 61s+ retreatment cases)	70	7	*M. fortuitum* *M. intracellulare* *M. chelonae*	Conventional biochemical methods	6.9	15.0	No	Previous TB
Nigeria	NA	Allana et al. (1991) [100]	NA	NA	NA	NA	*M. avium* *M. kansasii* *M. fortuitum*	Conventional biochemical methods	NA	NA	NA	NA

Nigeria	1963	Beer et al. (1965) [101]	≥1	NA	2682	149	Runyon 111 and 1V organisms	Conventional biochemical methods	6.0	NA	No	Previous TB
South Africa	2006-07	Clare et al. (2015) [29]	Median age-44	2496 presumptive TB cases	421	232	*M. kansasii* *M. gordonae*	Conventional biochemical methods	9.3	31.9	No	HIV infection
South Africa	1996-97	Corbett et al. (1999) [117]	NA	TB presumptive cases	NA	118	*M. kansasii* *M. fortuitum* *M. scrofulaceum*	Conventional biochemical methods	NA	34.0	Yes; 32	Previous TB, silicosis
South Africa	1993-96	Corbett et al. (1999) [38]	≥18	594 mine workers	NA	406 NTM	*M. kansasii* *M. fortuitum* *M. avium* complex	Conventional biochemical methods	68.4	13.1	Yes; 206	HIV infection, silicosis
South Africa	1993-96	Corbett et al. (1999) [119]	≥18	243 NTM infected suspects	92	243	*M. kansasii* *M. fortuitum* *M. intracellulare*	Conventional biochemical methods	100	NA	No	Previous TB, silicosis
South Africa	1993-96	Corbett et al. (1999) [118]	≥18	406 gold miners	NA	261 NTM patients	*M. kansasii* *M. scrofulaceum*	Conventional biochemical methods	64.3	NA	No	Previous TB, HIV infection
South Africa	2001-05	Hartherill et al. (2006) [120]	18 (13-23) months	1732 presumptive TB cases	94	109	*M. intracellulare* *M. gastri* *M. avium*	Molecular (RFPCR of 65 KD *hsp* gene)	6.3	4.2	Yes; 8	Previous TB

South Africa	2009	Sookan et al. (2014) [121]	NA	200 NTM suspects	NA	133 NTM patients	*M. avium* complex. *M. RGM* *M. gordonae*	Molecular (Genotype CM/AS assay)	66.5	NA	No	NA
South Africa	2008	Hoefsloot et al. (2013) [13]	NA	NA	NA	5646 NTM patients	MAC *M. kansasii* *M. scrofulaceum* *M. gordonae*	Molecular and biochemical methods	NA	NA	NA	NA
Tanzania	2012-13	Hoza et al. (2016) [9]	40 7-88	372 presumptive TB cases	85	36	*M. gordonae* *M. interjectum* *M. avium* complex *M. scrofulaceum*	Molecular (Genotype CM/AS assay)	9.7	33	No	HIV infection and age
Tanzania	2011	Haraka et al. (2012) [113]	35	1 HIV negative patient with prior TB	NA	1	*M. intracellulare*	Molecular (Genotype CM/AS assay)	100	100	Yes;1	Previous TB
Tanzania	2001-13	Katale et al. (2014) [112]	NA	472 presumptive TB cases	NA	12	*M. chelonae* *M. abscessus* *M. spaghni*	Molecular (Sequencing of *16S rRNA* gene)	2.5	NA	No	NA
Uganda	2009	Asimwe et al. (2013) [110]	12-18	2200 (710 infants and 1490 adolescents presumptive TB cases)	8	95	*M. fortuitum* *M. szulgai* *M. gordonae*	Molecular (Genotype CM/As assay)	4.3	NA	No	NA
Uganda	2012-13	Bainomugisa et al. (2013) [111]	NA	241 presumptive TB cases	95	14	*M. avium* *M. kansasii*	Molecular (Light cycler)	5.8	NA	No	NA

Zambia	2009-12	Mwikuma et al. (2015) [105]	NA	91 NTM suspected isolates	NA	54	*M. intracellulare* *M. lentiflavum.* *M. avium*	Molecular (Genotype CM/As assay)	59.3	NA	No	NA
Zambia	NA	Kapta et al. (2015) [104]	≥1	6123 presumptive TB cases enrolled	265	923	NTM not identified	Immunochroma tography assay (Capilia TAUNS method)	15.1	5.8	No	TB and HIV infection
Zambia	2001	Buijtels et al. (2010) [69]	≥15	167 chronically ill patients	74	93	*M. intracellulare* *M. lentiflavum* *M. chelonae*	Molecular (Sequencing of *16S rRNA* gene)	55.6	79.0	Yes; 7	Previous TB HIV infection
Zambia	2001	Buijtels et al. (2005) [69]	≥25	4 presumptive TB cases	NA	4	*M. lentiflavum* *M. goodie*	Molecular (Sequencing of *16S rRNA* gene)	100.0	33.0	No	HIV infection, damaged lungs
Zambia	2011-12	Malama et al. (2014) [107]	NA	100 presumptive TB cases	46	9	*M. intracellulare* *M. abscessus* *M. chimera*	Molecular (Sequencing of *16S rRNA* gene)	9.0	NA	NA	NA
Zambia	2002-03	Buijtels et al. (2009) [10]	≥15	565 (180 patients and 385 controls)	205	93	*M. intracellulare* *M. lentiflavum.* *M. avium*	Molecular (Sequencing of *16S rRNA* gene)	16.5	45.6	Yes; 1	Previous TB HIV infection, and use of tap water

Table 5: Non-tuberculous mycobacteria species isolated from sub-Saharan Africa, 1965-2015‡

Non-tuberculous mycobacteria species	Prior 2010 Biochemical identification methods	After 2010 Molecular identification methods	Previously associated with disease
M. intracellulare	Y	Y	Y
M. avium	Y	Y	Y
M. kansasii	Y	Y	Y
M. chelonae	Y	Y	Y
M. abscessus	Y	Y	Y
M. fortuitum	Y	Y	Y
M. scrofulaceum	Y	Y	Y
M. lentiflavum	Y	Y	Y
M. interjectum	Y	Y	Y
M. peregrinum	Y	Y	N
M. gordonae	Y	Y	N
M. xenopi	Y	Y	Y
M. malmoense	Y	Y	Y
M. moriokaense	Y	Y	N
M. kumamotonense	N	Y	N
M. kubicae	Y	Y	N
M. gordonae	Y	Y	N
M. simiae	Y	Y	Y
M. palustre	Y	Y	Y
M. indicus pranii	N	Y	N
M. elephantis	N	Y	N
M. flavascens	Y	Y	N
M. bouchedurhonense	N	Y	N
M. chimera	N	Y	Y
M. europaeum	N	Y	N
M. neoaurum	N	Y	N
M. asiaticum	Y	Y	N
M. nonchromogenicum	N	Y	N
M. gastri	Y	Y	N
M. nebraskense	Y	Y	N

M. confluentis	Y	Y	N
M. porcinum	Y	Y	Y
M. terrae	Y	Y	N
M. seoulense	Y	Y	N
M. engbackii	Y	Y	N
M. parascrofulaceum	Y	Y	N
M. triviale	Y	Y	N
M. scrofulaceum	Y	Y	Y
M. szulgai	Y	Y	Y
M. heckeshornense	Y	Y	N
M. poriferae	Y	Y	N
M. spaghni	Y	Y	N
M. goodie	Y	Y	N
M. aurum	Y	Y	N
M. conspicum	Y	Y	N
M. mucogenicum	Y	Y	N
M. rhodesia	Y	Y	N
M. gilvum	Y	Y	N
M. genevanse	N	Y	N
M. intermidium	N	Y	N
M. fortuitum 11/M. magaritense	N	Y	Y

Y=isolated N=not isolated

3.4.2 Synthesis of results

3.4.2.1 Epidemiology of Non-tuberculous Mycobacteria

The overall prevalence of NTM in pulmonary samples in sub-Saharan Africa derived from all 37 papers reviewed was 7.5% (95% CI: 7.2% - 7.8%). The median age of participants was 35 (Interquartile range, IQR 16 - 80) years based on 17 of 37 studies with age data. Majority (2325 [75.0%] of 3096) of subjects with NTM were males. Patients in 12 of 37 studies

(32.4%) had a previous history of pulmonary tuberculosis and only 15 (40.5%) studies investigated co-infection with HIV.

MAC species accounted for 28.0% (95% CI: 27.2 – 28.9%) of all NTM isolated and was the most frequently encountered NTM found in pulmonary samples in 19 of 37 studies. The prevalence of MAC ranged from 15.0% (95% CI: 5.05 – 25.0%) in Tanzania to 57.8% (95% CI: 36.3 – 76.9%) in Mali as shown in Figure 11 (along with country HIV prevalence in the legend [122]). There was regional variability in the distribution of NTM for example; 76.4% (95% CI: 74.8 – 77.9%) i.e. 2,355 of 3,084 MAC isolates from South Africa were *M. intracellulare,* while all MAC isolates from Mali were *M. avium.* Similarly, while *M. kansasii* was the third most isolated NTM in sub-Saharan Africa overall (4.7% [95% CI: 4.3 – 5.1%]), it was the top NTM in 5 (62.5%) of 8 studies in South africa. Other slow growing mycobacteria isolates, though less prevalent than MAC, were *M. scrofulaceum* 7.0% (95% CI: 6.4 – 7.5%) and *M. gordonae* 3.8% (95% CI: 3.4 – 4.3%). The rapidly growing mycobacteria i.e. *M. fortuitum, M. chelonae,* and *M. abscessus* accounted for just 1.2% (95% CI: 1.0 – 1.4%) of all NTM isolates from sub-Saharan Africa. Rapidly growing mycobacteria were reported predominantly from eastern African countries with *M. fortuitum* (43.0% [95% CI: 34.4 – 53.2%]) and *M. abscessus* (16.0% [95% CI: 9.4 – 25.9%]) as the top and second ranked NTM isolates from Uganda and Kenya respectively.

Among the 70.8% (6357 of 8980) fully speciated isolates in this review, there were 0.9% (56) *M. lentiflavum,* 0.9% (55) *M. malmoense,* 0.7% (43) *M. xenopi,* 0.4% (24) *M. gastri,* 0.3% (16) *M. szulgai,* 0.2% (15) *M. flavescens,* and 0.2% (11) *M. interjectum*. Unfortunately, 29.2% (95% CI: 28.1 – 30.1%) i.e. 2,623 of all 8,980 NTM isolates were not identified to species level.

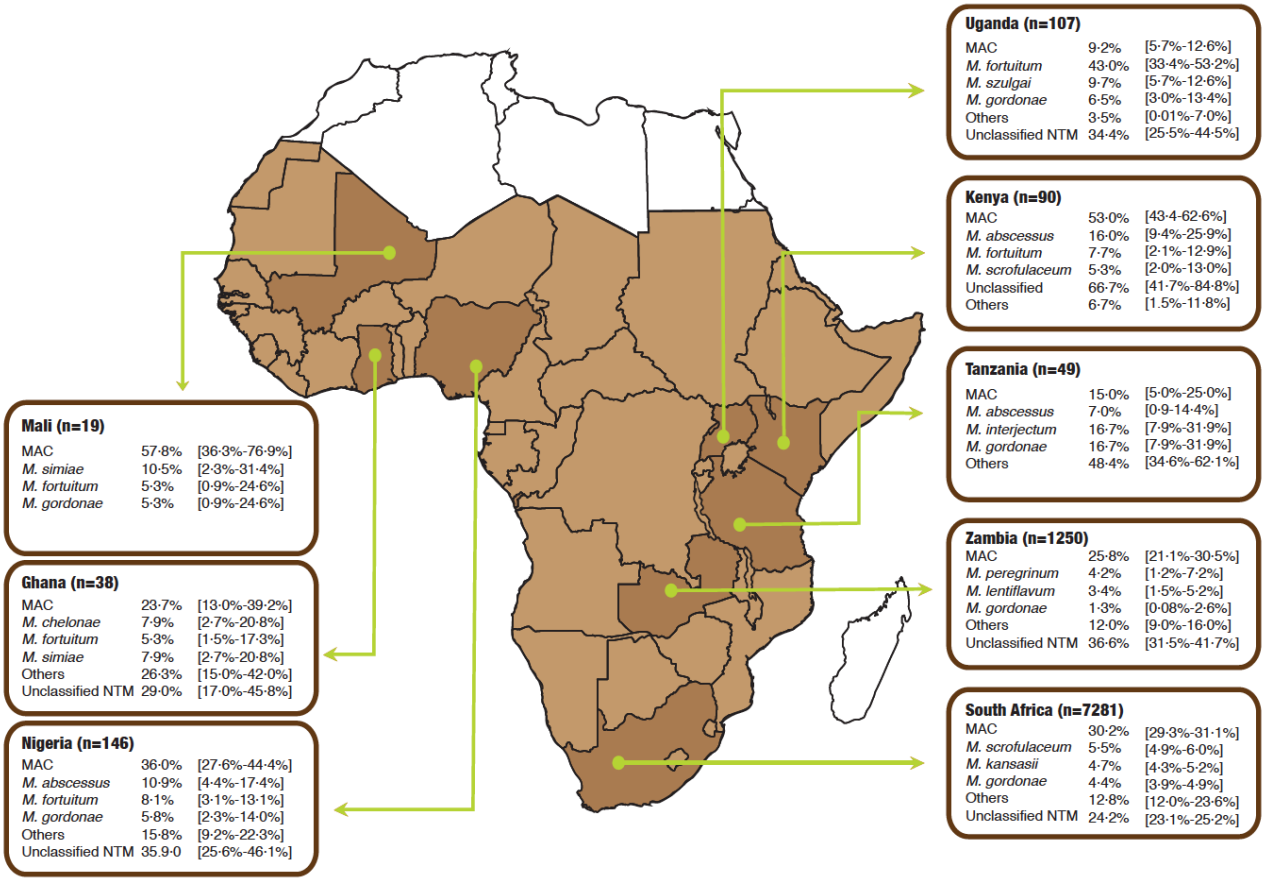

Figure 11: The distribution of the top four NTM identified from pulmonary samples with national HIV prevalence data: Mali (HIV 1.4%), Ghana (HIV 1.3%), Nigeria (HIV 3.1%), Uganda (HIV 7.1%), Kenya (HIV 5.9%), Tanzania (HIV 4.7%), Zambia (HIV 12.9%), and Republic of South Arica (HIV 19.2%), without taking into account of clinical relevance [123].

3.4.2.2 Pulmonary Non-tuberculous Mycobacteria Disease

Only 7 (19.0%) of the 37 articles investigated the clinical relevance of isolated NTM. Although these studies had 3,319 participants, only 962 (28.9%) had sufficient information to apply the ATS/IDSA criteria and of these, 266 (27.7%) met the definition of pulmonary NTM

disease. *M. kansasii*, isolated in 184 (69.2%) of 266 cases, was the most predominant cause of confirmed pulmonary NTM disease, followed by *M. scrofulaceum* (13.9%), MAC (13.5%), *M. lentiflavum* (1.9%), *M. simiae* (0.8%), *M. palustre* (0.4%) and *M. abscessus* (0.4%). Figure 12 shows the distribution of NTM species causing pulmonary NTM disease in sub-Saharan Africa by country. The studies investigating the clinical relevance of NTM isolates varied substantially in design, participant characteristics and background HIV prevalence (see Table 4). They ranged from a Zambian study that evaluated the clinical relevance of NTM isolated from 180 chronically ill patients and 385 healthy controls and found only 1.1% of isolates were clinically relevant [10] to a Malian study in patients with primary and chronic pulmonary tuberculosis where 57.9% of isolated NTM were clinically relevant [102].

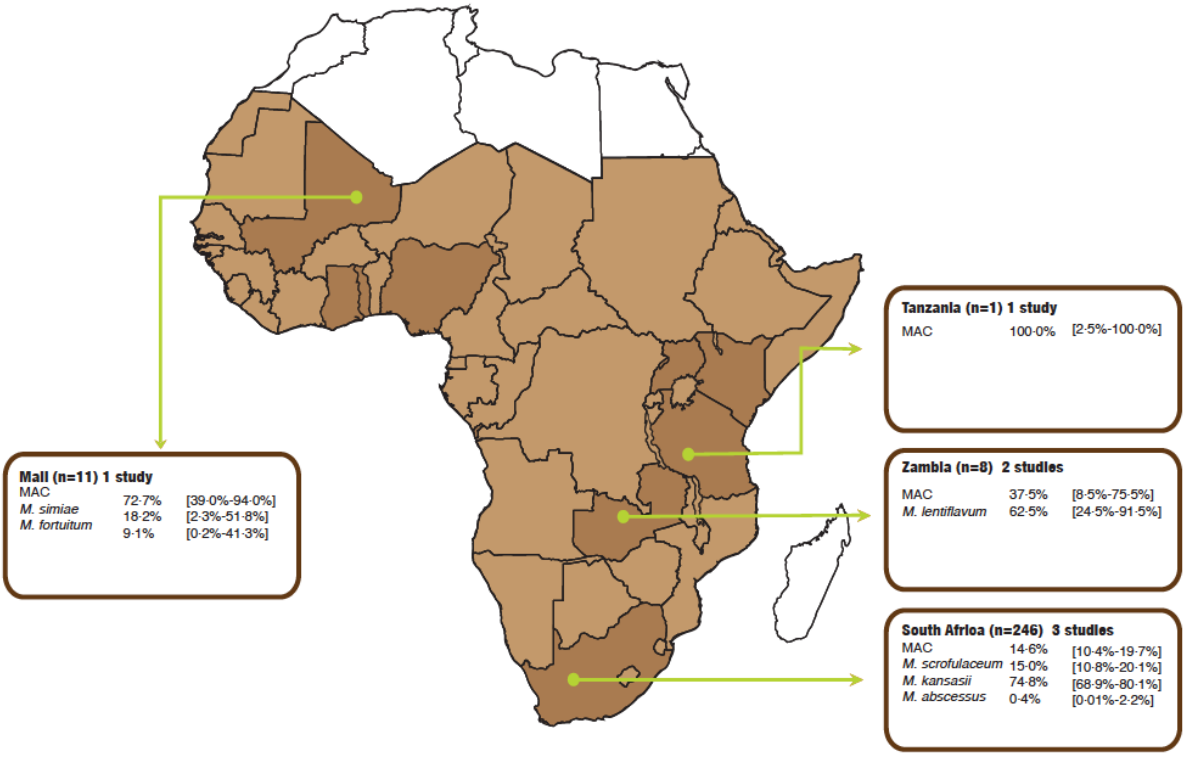

Figure 12: Non-tuberculous mycobacteria species causing pulmonary disease found in respiratory specimens in sub-Saharan Africa.

3.4.2.3 Clinical and Radiological Signs and Associated Morbidities

Of 3096 participants with NTM isolated, 80.7% (2498) and 87.5% (2,709) had clinical and radiological information respectively [38, 69, 79, 102, 113, 117, 120]. Clinical characteristics for NTM subjects closely mimicked those of pulmonary tuberculosis, as summarized in Table 4. There were radiological abnormalities in 79.0% (2141) of 2709 subjects, while 21.0% (568) had normal chest radiographs. Of the 512 with prior lung disease, 87.1% (446) had a history of tuberculosis and 12.9% (66) had bronchiectasis. In those with concurrent conditions, 50.2% (442) of 880 were co infected with HIV, 28.2% (248) reported

gastrointestinal diseases and 8.6% (76) complained of body weakness. Other characteristics are shown on Table 6.

Table 6: Clinical and radiographic characteristics for patients with pulmonary non-tuberculous mycobacteria regardless of clinical relevance in sub-Saharan Africa (n= 3096)

Characteristic	Numbers (%)
Clinical signs n=2, 498	
Cough ≥2 weeks	950 (38.0%)
Chest pain	684 (27.3%)
Significant weight loss	546 (21.9%)
Fever≥2	455 (18.2%)
Night sweats	211(8.4%)
Haemoptysis	27 (1.1%)
Dyspnoea	19 (0.8%)
Previous lung disease n= 512	
Bronchiectasis	66 (12.9%)
Tuberculosis	446 (87.1%)
Radiographic findings n= 2709	
Abnormal, suggestive of TB	1009 (37.2%)
No pathological changes	568 (20.9%)
Tuberculosis	446 (16.5%)
Nodules	203 (7.5%)
Fibrosis	140 (5.2%)
Cavitation	127 (4.7%)
Prior focal radiological scarring	107 (4.0%)

Bronchiectasis	66 (2.4%)
Abnormal, not consistent with TB	24 (0.9%)
Milliary TB	19 (0.7%)

Concurrent conditions n=880	
HIV infection	442 (50.2%)
Gastrointestinal disease	248 (28.2%)
Weakness	76 (8.6%)
Lymph node enlargement	52 (6.0%)
Splenomegaly	21 (2.4%)
Diabetes mellitus	22 (2.5%)
Hepatomegaly	19 (2.2%)

3.5 Discussion

We provide an overview of the epidemiology and geographical distribution of NTM species isolated from pulmonary samples in sub-Saharan Africa. To our knowledge, this is the first comprehensive review of pulmonary NTM in this part of the world. Similar to reviews by other authors, our findings suggest diversity in prevalent NTM species, geographical variation in NTM distribution and their clinical relevance across the sub-continent [37].

The global collection of NTM isolated from pulmonary samples reported by Hoefsloot et al. [13] in 2008 included isolates from only one sub-Saharan Africa country, South Africa. The update in 2013 by Kendall et al. did not improve significantly on the earlier review with respect to additional African NTM isolates [7]. Despite the relative scarcity of local data, it is important to highlight that this review is the first to include NTM data for nine countries in sub Saharan Africa.

Overall, we found a predominance of MAC from pulmonary samples in countries of Western, Eastern and Southern Africa. *M. scrofulaceum* and *M. kansasii* were predominant in Southern Africa and the rapidly growing mycobacteria (*M. abscessus*, *M. fortuitum* and *M. chelonae*) in Eastern Africa. These findings are consistent with the predominance of MAC in the epidemiology of NTM in North America [2, 7, 67] Europe [124] Australia [125] and East Asia [31]. The relative preponderance of the two members of the MAC family also varied by region with *M. intracellulare* predominating in South Africa while all MAC isolates from Mali were *M. avium*. However, the South African study had a much bigger sample size compared to the Malian study. While MAC was the most frequently implicated NTM in colonisation, *M. kansasii* was the most common in pulmonary NTM disease. The dominance of *M. kansasii* as well as the presence of *M. scrofulaceum* in South Africa was speculated to

be linked to mining activities and significant urbanisation in the country, resulting in a socio-economically disadvantaged population [28, 33, 126] working in the mines, and frequently suffering from silicosis, while living in poor, overcrowded environments..

When the South Africa pulmonary NTM data is excluded, MAC is the major cause of pulmonary NTM as reported in North America, Europe, Australia and Asia [7]. Because relatively few studies in this review applied the ATS/IDSA criteria for confirmation of pulmonary NTM disease, it is difficult to reach conclusions regarding the dominant NTM species causing pulmonary disease in sub-Saharan Africa.

In contrast to observations from other parts of the world, especially in Europe, where *M. malmoense* and *M. xenopi* are well known for causing pulmonary NTM disease [2, 27] these NTM were not represented in the limited number of studies reviewed here. *M. xenopi* was rare in sub-Saharan Africa which is not unexpected considering its association with hot water delivery systems that are less developed in sub-Saharan Africa compared to industrialised countries.

Pulmonary NTM was commonly associated with a history of previous pulmonary tuberculosis in sub-Saharan Africa compared to Europe and North America. This is not surprising given the high incidence of MTBC disease in sub-Saharan Africa [127]. Pulmonary tuberculosis is associated with significant sequelae that have not been adequately studied in sub-Saharan Africa. The associated structural lung damage, chronic pulmonary obstructive disease most likely favour colonization by NTM and other pathogens [2]. It is also likely that the increasing isolation of NTM has come from investigation of patients with chronic pulmonary disease including those complicating previous pulmonary tuberculosis

[53, 102]. In light of this, the clinical, radiological and microbiologic criteria of the ATS\IDSA is important for distinguishing colonization from pulmonary NTM, particularly in sub-Saharan Africa that is endemic for MTBC [55].

Many rarely isolated NTM were also identified in presumptive tuberculosis patients, for example *M. genavense*, *M. gilvum*, *M. intermedium*, *M. poriferae*, *M. spaghni*, *M. interjectum*, *M. peregrinum*, *M. moriokaense*, *M. kumamotonense* and *M. kubicae.* Although some of these species have also been isolated in other parts of the world from pulmonary samples in patients with chronic bronchitis, pulmonary tuberculosis, sub-acute pneumonia and healed tuberculosis[128, 129] it is currently unclear what role they play in the aetiology of pulmonary disease in Africa.

The HIV-driven increase in the risk of tuberculosis disease in sub-Saharan Africa has been well described and for NTM, MAC is a particularly well described opportunistic infection in patients with AIDS. We found almost half of all cases of confirmed pulmonary NTM were also HIV co-infected. This suggests the possibility of HIV attributable pulmonary NTM beyond the now familiar disseminated MAC disease often seen in persons with AIDS.

Persons with pulmonary NTM disease in sub-Saharan Africa are younger than observed in North America, Europe and Australia where increasing age (≥50 years), structural lung damage, immunosuppressive chemotherapy for cancer, autoimmune and rheumatoid conditions are the most frequently reported risk factors for this disease [2, 7, 39, 42]. Given the younger age and higher burden of pulmonary tuberculosis and HIV co-infection in sub-Saharan Africa, it is not surprising that we found pulmonary NTM disease mostly in the 33-44 year-age group.

Our review has a number of limitations: we only searched for English language-articles. Given the numbers of Francophone countries in sub-Saharan Africa, French-language publications may have been missed. In addition, our assessment of the clinical relevance of isolated NTM was not as comprehensive as desired because majority of the studies did not collect the detailed clinical, radiological and microbiological data required to do this. We also could not report the full diversity of NTM in colonization and disease because almost 30% of all isolates were not fully identified to species level. Since the studies reviewed came from varied time periods during which laboratory procedures for ascertainment differed, we cannot exclude the possibility of laboratory procedures before and/or after year 2000 selecting for particular NTM species whilst inhibiting others [79]. For example, the wider usage of sensitive liquid culture media could in theory have selected for specific NTM species. Similarly, the increasing use of molecular methods for identification of current and historical isolates, especially for the MAC and rapidly growing mycobacteria groups, could underpin the changes to NTM taxonomy over time [22, 130, 131]. However, we think our results were not significantly affected because the distribution of NTM species identified in the periods before and after 2010 were similar. It was also not possible to link the pulmonary NTM reported to prevalent NTM species in the environment so as to reach conclusions regarding the geographical variation in NTM because data on environmental NTM in sub-Saharan Africa is even more sparse than for human studies. Given the heterogeneity of studies included in this review including laboratory methods and quality standards, some of the NTM reported here may be due to contamination especially for NTM like *M.flavescens* that are frequent laboratory contaminants. It is possible for example that all 7 *M.flavescens* reported were contaminants. In more than half of 26 studies that used molecular techniques to identify

NTM, 16s rDNA sequencing was used. However, this method has a limitation in that it is not fully capable of distinguishing between all the different NTM species for example *M. abscessus* and *M. chelonae*. Therefore, it is possible some species have been misidentified or misclassified in these studies.

To conclude, we have provided the first detailed review of pulmonary NTM in sub-Saharan Africa. This review highlights the contribution of NTM to the aetiology of tuberculous-like pulmonary disease in the sub-continent. It also suggests their presence as commensals in pulmonary samples may confound the diagnosis of pulmonary tuberculosis, especially in those with previous history of tuberculosis and/or other chronic respiratory conditions.

Additional research and surveillance is required for investigation of the full contribution of NTM to pulmonary disease, to describe the full repertoire of prevalent and incident NTM, and to determine the role of risk factors (particularly HIV/AIDS) for colonization and/or disease. Given the risk of over diagnosis of NTM in pulmonary samples as tuberculosis disease, resulting in repeated courses of treatment in previously treated tuberculosis patients, investments in and development of point of care diagnostics for NTM are required.

4 Epidemiology of Non-tuberculous Mycobacteria in The Gambia-Pulmonary Carriage and Disease

During the nationwide Gambian Survey of Tuberculosis Prevalence (GAMSTEP) that described the population prevalence of pulmonary MTBC disease [132], significant numbers of AFB positive sputum cultures that were negative for MTBC [according to the BD MGIT ™ TBc Identification (Becton, Dickinson and Company, USA) rapid test] were considered suspicious for NTM. The TB prevalence survey at the Medical Research Council Unit The Gambia (MRCG), provided a unique opportunity to estimate the population prevalence of NTM in pulmonary samples, determine the prevalence of confirmed/clinical pulmonary NTM disease and investigate associated risk factors in the general population.

This is the first study in The Gambia to report on NTM species isolated from pulmonary samples and their clinical relevance.

4.1 Study Hypothesis

Non-tuberculous mycobacteria are prevalent in sputum samples of a population sample in The Gambia and can cause pulmonary NTM dsease..

4.2 Aims of Study

The overall aim of the study is to estimate the prevalence and predictors of NTM in colonisation and disease in pulmonary samples of a subset of The Gambia population.

4.3 Primary Objectives:

1. To speciate suspected NTM from sputum samples of a representative population of The Gambia using the *16S rRNA* gene sequencing analysis technique.
2. To determine the prevalence, and explore the pattern of occurrence, of NTM in four participant groupings with the following features:
    - No respiratory symptoms and abnormal CXR
    - No respiratory symptoms and normal CXR
    - Respiratory symptoms and abnormal CXR
    - Respiratory symptoms and normal CXR
3. To explore the risk factors (e.g. age, gender, clinical and radiological features, urban versus rural residence and history of previous pulmonary TB) for NTM colonisation and disease.
4. To explore the clinical relevance of isolated pulmonary NTM using the American Thoracic Society/Infectious Disease Society of America diagnostic criteria for pulmonary NTM disease.

4.4 Materials and Methods

4.4.1 Ethical Approval

The MRCG Scientific Coordinating Committee (SCC) and the MRCG/Gambia Government Joint Ethics Committee approved this study (SCC number 1371).

4.4.2 Study Area, Subjects and Samples

Following prerequisite SCC and ethical approvals for Gambian Survey of TB Prevalence, all fieldwork, including collection of demographic and clinical data (including CXR findings),

and laboratory work were completed at the MRCG TB diagnostic and research molecular biology laboratories as already described [132]. This MPhil project started with retrieval of archived sputa from the freezer and the parent study, the Gambian Survey of TB Prevalence, was not part of my MPhil.

On conclusion of the parent survey, 903 suspected cases of NTM were identified from decontaminated sputa that yielded AFB positive culture yet were negative for MTBC following testing with the rapid identification assay, BD MGIT ™ TBc Identification (Becton, Dickinson and Company, USA). This rapid identification assay tests for the MTBC-specific *Mycobacterium* Protein Target 64 (MPT64) antigen. All 903 were selected for inclusion in this study. However, following retrieval of stored sputa, only 575 (63.7%) of 903 samples yielded AFB positive cultures, all of which were selected for analysis.

This study population was categorized into 4 groups as shown in Table 7. The second category of participants screened positive for CXR features suggestive of TB during field screening in the parent study but were subsequently re-classified as normal by an expert radiology panel. As a result, they became a convenient control group (asymptomatic for TB and normal CXR) for this project. I was blinded to these categories while working on the specimens.

Table 7: Categorization of suspected NTM study participants

Category	Classification of Participants	Number of available participants with suspected NTM cultures	
		GAMSTEP (903)	NTM study (575)
1	No respiratory symptoms and abnormal CXR	408	266
2	No Respiratory symptoms with a normal CXR	22	16
3	Respiratory symptoms and abnormal CXR	199	133
4	Respiratory symptoms and normal CXR	274	160
Total		903	575

4.5 Laboratory Methods

4.5.1 Smear Microscopy, Decontamination and Liquid Cultures as part of Gambian Survey of Tuberculosis Prevalence (GAMSTEP) Activities

Fluorescent microscopy was performed on all sputum samples with 0.5% Auramine stain in the parent study. The sputa were then decontaminated by treating them with 1.0% N-acetyl-L-cysteine (NALC), 4.0% sodium hydroxide and 2.9% sodium citrate. They were incubated in the BACTEC MGIT 960™ system (Becton Dickinson Diagnostic Instrument Systems) as described by Rodrigues *et al.* [133]. Samples without any growth after 42 days of incubation were removed and classified as negative.

Samples with growth were removed from the machine and inoculated on blood agar to check for contamination. Microscopy with ZN staining was then performed to check for the presence of AFB. Non-AFB samples were considered contaminants and excluded from the study. BACTEC MGIT positive cultures that tested positive for AFB on ZN microscopy were

further tested with the TBc rapid antigen assay to confirm MTBC. Where MTBC were confirmed, cultures were further sub-cultured onto Lowenstein Jensen (LJ) media. Suspensions were made from matured colonies in tryptone soya broth (soybean casein digest medium) and stored at -70^{o}C [132].

4.5.2 NTM Identification and Characterization

4.5.2.1 Culture of Decontaminated Sputum Samples

In the parent study, AFB positive IBD rapid test negative cultures were not stored. I therefore had to use the archived, decontaminated sputa from which these suspected NTM cultures were originally identified for this work. MGIT tubes containing 7mL of modified Middlebrook 7H9 broth base were used for the detection and recovery of NTM. These tubes were supplemented with 0.8mL of BACTEC^{TM} MGIT^{TM} growth supplement (to provide substances essential for the rapid growth of mycobacteria) and BBL^{TM} MGIT^{TM} PANTA^{TM} antibiotic mixture (to inhibit growth of non- mycobacterial agents) before inoculation with 0.5mL of stored, decontaminated sputum samples from which suspected NTM were identified in GAMSTEP. MGIT tubes were incubated at 37^{o}C in the automated BACTEC MGIT 960^{TM} system (Becton Dickinson Diagnostic Instrument Systems) until flagged as positive or negative after 42 days. All AFB positive cultures were classified as suspected NTM while non-AFB cultures were considered contaminants and excluded from the study.

4.5.2.2 16S Ribosomal Ribonucleic Acid (*16S rRNA*) gene PCRs

In preparation for *16S rRNA* gene PCRs, mycobacterial DNA was extracted from MGIT cultures by the boiled lysate method described by Wade et al [134]. Briefly, 0.5 mL of culture was centrifuged at 10,000 rpm for 15 minutes.

The resulting pellets were resuspended in 0.1mL sterile Tris ethylene diamine tetra acetic acid (TE) buffer® (Sigma Aldrich, St. Louis, Missouri, United States) and suspensions heated in a heat block at 99 ºC for 20 minutes followed by sonication for 15 minutes. Tubes were then spun at 14,000 rpm for 5 minutes following which 50 µl aliquots of the supernatant were transferred to sterile tubes and stored at - 20°C until PCRs were performed.

4.5.2.3 Primers

With standard mycobacteria genus-specific primers (Metabion international Martinsried/ Deutschland), a 960 bp sequence of the mycobacterial *16S rRNA* gene was amplified as earlier described by Böddinghaus et al. [135]. Concentrated stock aliquots (100 µM) of primers P1 and P2 new [136]. were reconstituted with nuclease-free water. For working solutions, stock primers were diluted 10 times (1:10 dilutions) to give a 10 µM primer working concentration, then both stock and working solutions were stored at -20°C (Table 8).

Table 8: Primers for NTM identification in study

Primer names	Sequence	Melting temperature (Tm)	Expected amplicon band size (bp)
P1 (Forward primer)	5'-TGCTTAACACATGCAAGTCG-3'	56°C	~ 960bp
P2 new (Reverse primer)	5'-TCTCTAGACGCGTCCTGTGC -3'	63°C	~ 960bp

4.5.2.4 Optimization Experiments and *16S rRNA* gene Amplification

Optimal reagent/solution concentrations, assay amplification conditions (cycling temperatures/numbers) and equipment required to amplify the mycobacterial 16S rRNA gene were determined prior to NTM identification and characterization. In these experiments, various primer concentrations of P1 and P2 new (0.08 µM, 0.2 µM 0.35 µM) were tested

against an annealing temperature gradient of 52ºC to 60ºC. A 2.5 µL aliquot of DNA from a mycobacterial reference strain (*M. tuberculosis* $H_{37}RV$) was used in a total reaction volume of 25 µL containing 2.5 µL of 10X buffer (containing 1.5 mM magnesium chloride solution), 0.5 µL of 10 mM deoxyribonucleotide triphosphate (dNTPs), 0.08 to 0.35 µL of 10 µM P1/P2 primers, and 0.2 µL of 5.0 U/µl of *Taq* DNA polymerase (Qiagen).

The optimal PCR conditions identified were: one cycle of denaturation at 95°C for 5 minutes, 45 cycles consisting of denaturation at 95°C for 45 seconds, annealing at 52°C to 60°C for 45 seconds, an extension at 72°C for 45 seconds and a final cycle of extension at 72°C for 10 minutes. Amplification was carried out in a 96-well microtiter plate using a gradient thermocycler. Gel electrophoresis was carried out for detection of PCR amplification products on a 1% (w/v) agarose gel. The set of amplification conditions which produced the brightest, single amplicon band of ~960 bp were chosen as the optimal PCR conditions for this project. PCRs were performed in a 25 µl reaction volume under the PCR conditions outlined on Table 9. All PCR products were stored at -20 ºC for further laboratory processin

Table 9: *16S rRNA* gene PCR reaction mix

Reagent/solution	Volume (µl) for one reaction	Final Concentrations
PCR water	18.9	
10X buffer $MgCl_2$ (1.5 mM)	2.5	1X
dNTPs (10 mM)	0.5	0.2 mM
Primer F (10 µM)	0.2	0.08 µM
Primer R (10 µM)	0.2	0.08 µM
Taq (5 U/µl)	0.2	0.04 U/µl
gDNA (ng)	2.5	
Total	25	

4.5.2.5 Detection of PCR Amplification

Agarose gel electrophoresis was used to check if desired PCR products (amplicons) were amplified and to estimate amplicon sizes. Five microliter of PCR products were loaded on 1% (w/v) agarose gel slabs stained with ethidium bromide (5 µg/ml) and analysed by gel electrophoresis in 1 x TAE buffer (40 mM Tris, 20 mM of glacial acetic acid, 1 mM EDTA, pH 8.0) for 35 minutes at 100 Volts. The size of amplicons was estimated by comparison with 1 Kbp DNA ladder (*BioLabs*, New England) and visualized using Bio-Rad Molecular Imaging System (USA).

4.5.2.6 Purification of PCR Products and Sequencing

Amplicons from suspected NTM cultures amplified as mycobacteria were cleaned using exonuclease and shrimp alkaline phosphatase (EXOSAP) to remove nonspecific PCR products. Briefly, 1µl of shrimp alkaline phosphatase and 1 µl of exonuclease were added into a sterile 1.5 ml micro centrifuge tube containing 10 µl of nuclease-free water. The suspension was mixed by a gentle tap and then 10 µl dispensed into wells of a 96 well plate. 10 µl samples of each amplicon was dispensed into a well and mixed by up and downward pipetting using filter-barrier pipette tips. The plate was sealed with an adhesive film and spun briefly. EXOSAP reaction was carried out in a thermal cycler in an initial step at 37°C for 15 minutes followed by another step at 80°C for 20 minutes. Cleaned PCR products were stored at -20°C until sequencing reactions were carried out.

Cycle sequencing reactions were done in 10 µl reaction volumes containing 4.36 µl nuclease-free H_2O, 2 µl of 5X sequencing buffer, 0.64 µl of 10 µM P1 and P 2 primer each, 0.5 µl Big Dye (Applied Biosystems (ABI) terminator kit version 3.1 chemistry) and 2.5 µl of cleaned PCR product. PCR products from the cycle sequencing reaction were further purified using Sephadex® G-50 columns (Sigma-Aldrich). Briefly, G-50 powder was added onto a Multiscreen Column Loader and sprayed over to fill in the number of wells that were needed using a Multiscreen Loader scraper. 300 µl of nuclease free water was added into each well and allowed to sit at room temperature for two hours and then centrifuged at 2300 rpm for five minutes to remove water from the G-50 column. The column was washed twice with nuclease-free water and centrifuged as described above. The G-50 plate was transferred to a sequencing plate and 11 µl of PCR product added to appropriate wells. The plate was centrifuged at 2300 rpm for five minutes to filter out the PCR products into their

corresponding wells on the sequencing plate. The products were dried in a thermal cycler for 20 minutes at 90°C. 10 µl of formamide was added into each well containing dried product. The plate was vortexed briefly, centrifuged briefly and products denatured at 90°C for 2 minutes. The Sanger sequencing method using the ABI 3130x DNA analyser was used to determine the order of nucleotide sequence in the PCR products.

4.5.2.7 Editing Sequence Data and NTM Identification

Background and ambiguous non-standard nucleotides were removed and the correct DNA nucleotides inserted. Unknown sequences were edited in BioEdit software (version 7.2.5 Ibis Biosciences). Consensus sequences were computed by matching forward and reverse traces of each sample using SeqTrace 0.9.0 software. Filtering of low quality base calls and end trimming were also performed and high quality finished unknown sequences were exported in Fasta format for downstream analyses.

To illustrate phylogenetic relationship of unknown sequences and subsequently identify them, NTM and MTBC *16S rRNA* reference sequences derived from American Type Culture Collection (ATCC isolates) were downloaded from Gen Bank [137]. These sequences were aligned together with unknown sequences in BioEdit software using ClustalW2 Multiple Sequence Alignment tool. A Phylogenetic tree was constructed from the aligned sequences based on regions A and B of the Mycobacteria gene using maximum likelihood algorithm implemented by PhyMl (PHYlogenetic inferences using Maximum Likelihood). Unknown sequences were further identified based on their relatedness to NTM and MTBC reference strains on phylogenetic tree.

4.6 Quality Control Measures for Preventing Contamination

Contamination during specimen analysis leading to false positive results can be common [2, 27]. This phenomenon is typically caused by contamination of the specimen with NTM contained in non-sterile laboratory water used for preparation of reagents and solutions and/or cross contamination from a heavily infected specimen to other specimens being analysed at the same time. To avoid this kind of contamination, quality control (QC) measures were put in place during processing of all suspected NTM cultures to detect and exclude environmental NTM contamination. These included the following:

- Aseptic mycobacteriological techniques such as use of aerosol barrier pipette tips, changing pipette tips between all liquid transfers, pulse centrifugation of tubes after vortexing to remove drops from inside of the lid and working in a biological safety cabinet. Hand gloves were worn throughout laboratory procedures and these were duly changed whenever they encountered any sample.
- All water used was ultrapure, sterile-filtered, deionised water. Untreated laboratory water was NEVER used in any of the assays. All solutions/reagents employed for the isolation and culture of mycobacteria were sterilized either by autoclaving or filtration and quality controlled prior to usage.
- For DNA extraction, negative controls (nuclease-free water) were included in each batch of extraction procedure to exclude NTM contamination of laboratory reagents/ solutions and cross contamination.
- For the *16S rRNA* gene PCRs and sequencing, non-template controls (NTC) were included in each batch of pre- and post-PCRs to exclude NTM contamination from laboratory reagents, solutions and the environment.

- An audit was performed in our laboratory to screen for possible sources of laboratory contamination. Samples of laboratory-distilled water, and all reagents and solutions used for identification and characterization of NTM were cultured and subjected to *16S rRNA* gene PCR.

4.7 Analysis

All data analyses for this study were carried out with Stata version 12 (Stata Corp, College Station, TX) with the help of MRCG Statistician. The main outcome of the study was the prevalence of NTM in the study population. NTM prevalence and other categorical variables were summarised using frequency counts and proportions. From the samples selected from the parent study, there were 328 participants with culture negative results compared to the original study. NTM prevalence was estimated using 2 models. **Model 1:** Complete case analysis - Here I analysed only samples that had a complete data set for all variables. **Model 2:** Here I assumed the results for the 328 participants with negative cultures were truly negative. Results from the 2 models are presented.

Associations between NTM prevalence and categorical risk factors were examined in univariate analysis using mixed-effects logistic regression . Those reaching statistical significance ($p<0.05$) in univariate analyses were included in random effects multivariate logistic regression models to obtain odds ratios and their 95% confidence intervals adjusted for risk factors and clustering in the design of the parent study. Estimates derived from regression models were adjusted for urban/rural residence, gender, age and ethnicity.

Participants with NTM positive samples were assessed for clinically relevant disease using the ATS/IDSA diagnostic criteria and classified as confirmed, probable, suspected and non-pulmonary NTM disease.

4.8 Results

4.8.1 Outcome of Optimisation Experiments

Here, results of the initial optimisation and quality control for experiments for NTM identification, characterisation and results of *16S rRNA* gene sequencing analyses are presented.

Of the three primer concentrations (0.35 µM, 0.2 µM and 0.08 µM) used against a range of annealing temperature gradient of 52^{o}C to 60^{o}C, only primer concentrations of 0.08 µM yielded clear, sharp and bright amplification products with expected size of 960bp as shown in Figure 13. Sequencing of the PCR products also correctly identified NTM, positive and negative controls. Therefore, 0.08 µM primer concentrations with 56^{o}C annealing temperatures were adopted as optimal conditions for *16S rRNA* gene PCRs.

4.8.2 Quality Control

The quality control measures implemented were successful in preventing contamination because cultures of samples of distilled water, reagents and solutions used for processing and identification of NTM in our laboratory did not yield any NTM growth. In addition, PCR on these reagent samples did not amplify any DNA (see Figure 14).

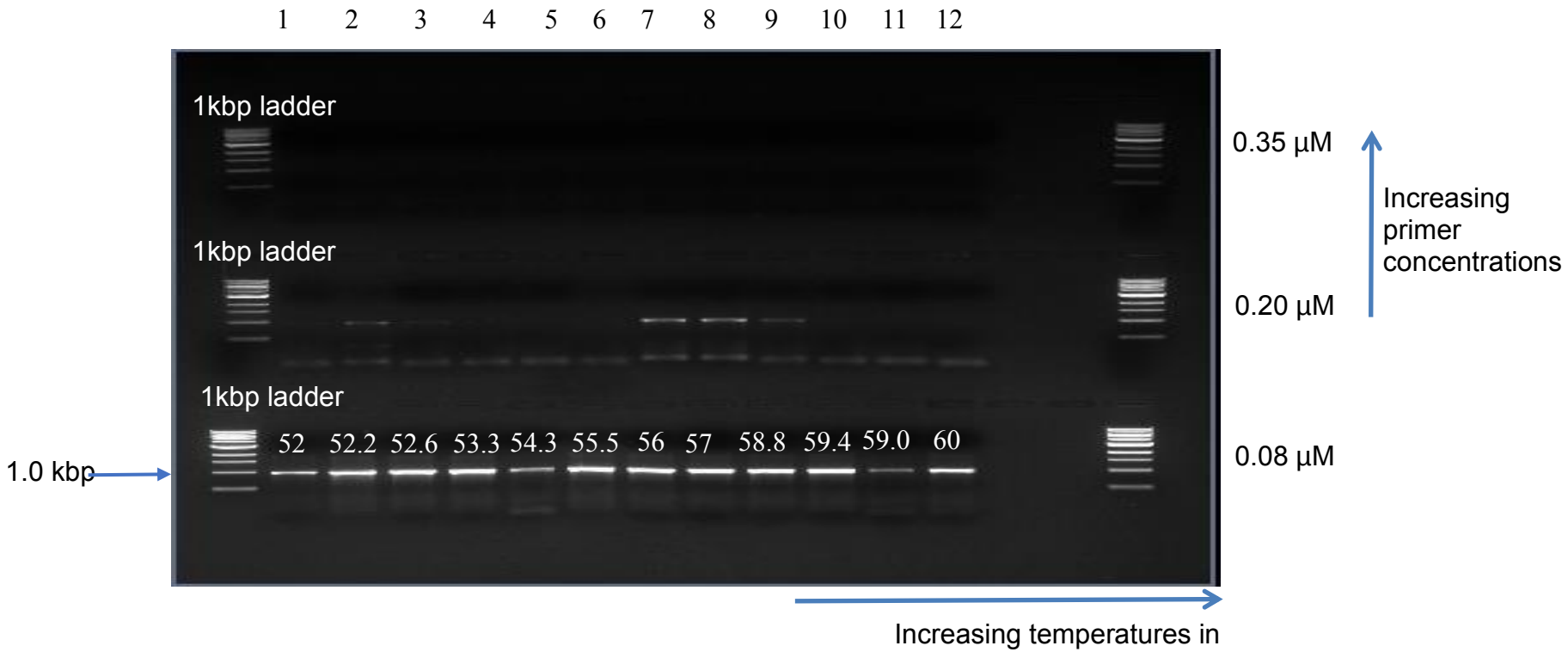

Figure 13: Outcome of PCR optimization experiments

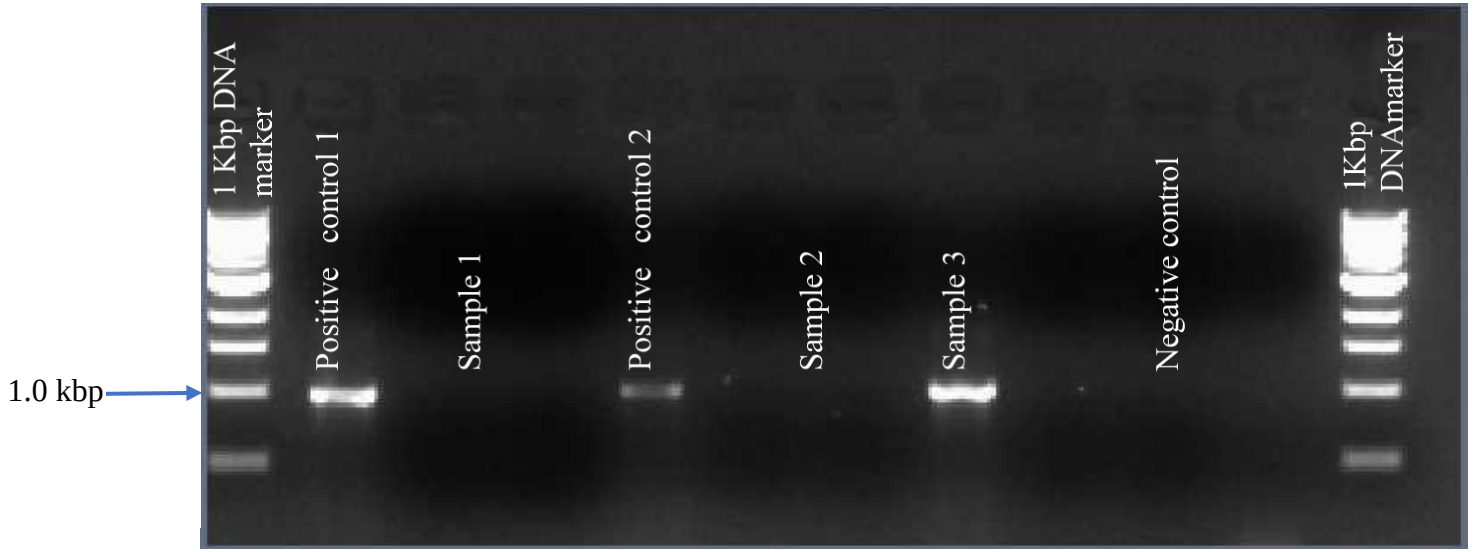

Figure 14: Quality control on positive and negative controls, reagents and solutions used for 16S rRNA gene PCRs. DNA marker - 1 Kbp DNA marker; positive control 1- H37RV; positive control 2- BCG; sample 1- TE buffer; sample 2- laboratory distilled water; sample 3- *Mycobacterium avium* complex; negative control - *Escherichia coli*; TE -Tris ethylene diamine tetra acetic acid; Kbp - Kilo base pair; DNA - Deoxyribonucleic acid

4.8.3 Demographic Characteristics of Participants

The socio-demographic characteristics of the study population are displayed in Table 10. Total participants, 575 as earlier indicated provided 730 samples as shown in the CONSORT diagram - Figure 15.

The median age (Interquartile range-IQR) of participants was 55 (IQR 40 – 70) years. More than half were ≥55 years old. The majority of the subjects were female and lived in rural areas of The Gambia.

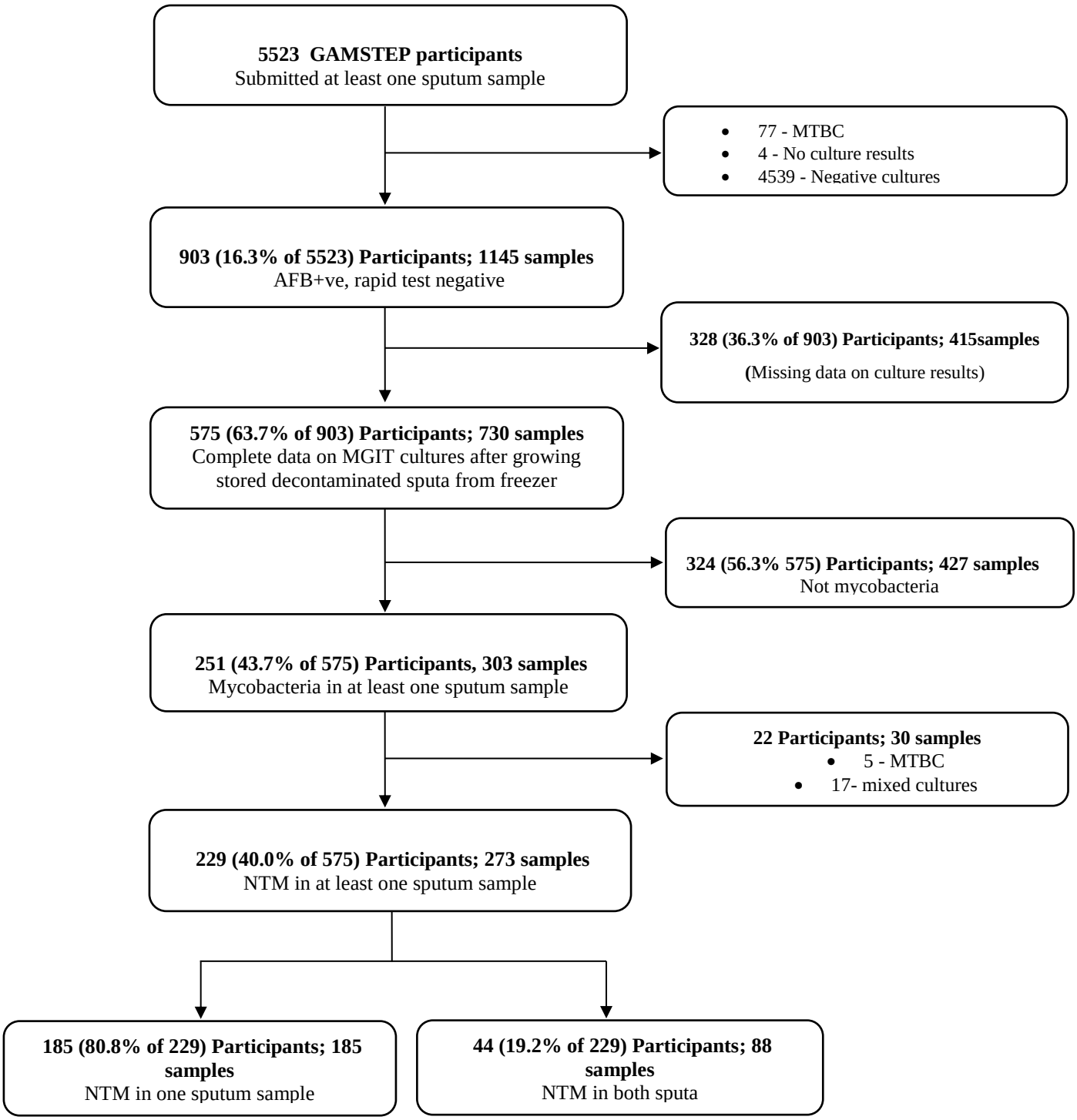

Figure 15: Outline of the non-tuberculous mycobacteria study in The Gambia

Table 10: Socio-demographic Characteristics of the Study Population (n=575)

Characteristics	Presumptive NTM cases (n=575) (%)	Positive for NTM (n=229) (%)
Gender		
Male	246 (42.8)	88 (38.4)
Age group (years)		
15-24	41 (7.1)	7 (3.1)
25-34	42 (7.3)	19 (8.3)
35-44	84 (14.6)	38 (16.6)
45-54	100 (17.4)	39 (17.0)
55-64	106 (18.4)	38 (16.6)
≥65	202 (35.1)	88 (38.4)
Clinical symptoms		
Fever	260 (45.2)	107 (46.7)
Cough	227 (39.5)	78 (34.1)
Night sweats	64 (11.1)	29 (12.7)
Prior tuberculosis	40 (7.0)	12 (5.2)
Ethnicity		
Mandinka	216 (37.6)	79 (34.5)
Wolof	100 (17.4)	54 (23.6)
Fula	139 (24.2)	57 (24.9)
Others	120 (26.9)	39 (17.0)
Region of country		
West Coast	109 (19.0)	19 (8.3)
Central River	163 (28.4)	93 (40.6)

Greater Banjul	97 (16.8)	29 (12.3)
North Bank	101 (17.6)	50 (21.8)
Upper River	83 (14.3)	35 (15.3)
Lower river	22 (3.8)	3 (1.3)
Location		
Rural	400 (69.6)	172 (75.1)
Urban	175 (30.4)	57 (24.9)
Occupation		
Professional	70 (12.7)	21 (9.2)
Service worker	30 (5.2)	10 (4.4)
Agriculture	222 (38.6)	93 (40.6)
Dependant	247 (43.0)	104 (45.4)
Others	6 (1.0)	1 (0.4)
Education		
Read in English/Arabic	511 (88.9)	214 (93.5)
Primary	41 (7.1)	11 (4.8)
High school/Diploma/University	23 (4.0)	4 (1.8)
Chest X ray		
Normal	191 (33.2)	77 (33.6)
Abnormal	384 (67.2)	152 (66.4)
Participant categories		
No respiratory symptoms; abnormal CXR	266 (46.3)	103 (45.0)
No respiratory symptoms; normal CXR	16 (2.3)	8 (3.5)
Respiratory symptoms; abnormal CXR	133 (23.1)	48 (21.0)
Respiratory symptoms; normal CXR	160 (27.8)	70 (30.6)

4.8.4 Pulmonary NTM Prevalence and Risk Factors

The estimated prevalence of NTM in pulmonary samples derived from the 2 approaches are shown in Table 11. As expected, the lowest prevalence estimate is seen with the most conservative model (model 2). The assumption underpinning model 2 is the least realistic and provides justification for disregarding this prevalence estimate. The prevalence estimate from model 1 in this case is the better option because it uses most of the available data.

Table 11: Estimated overall prevalence of NTM in pulmonary samples in The Gambia

	Model 1 (N=575)	Model 2 (N=903)
NTM prevalence estimate	39.8% (95% CI: 35.8% - 44.0%)	12.8% (95% CI: 8.8% - 18.4%)

Model 1 = Complete case analysis (analysis of samples that had a complete data for all variables); model 2 = Assuming negative data for 328 negative cultures in present (NTM) study

As shown in Table 12, with stratification into the different participant categories, the group without respiratory symptoms and normal CXR had the highest proportion of NTM positive participants compared to other groups. However, these observed differences were not statistically significant (test of difference in proportions, z = -1.1944 p=0.23). This is supported by the relatively wide and overlapping 95% CIs.

Table 12: Prevalence of NTM across four participant groups in study population n=229

Participant categories	Total (N)	Positive n (%)	95%CI
No respiratory symptoms; abnormal CXR	266	103 (38.7)	32.8 – 44.7
No respiratory symptoms, normal CXR	16	8 (50.0)	24.7 – 75.3
Respiratory symptoms, abnormal CXR	133	48 (36.1)	28.0 – 44.9
Respiratory symptoms, normal CXR	160	70 (43.8)	36.0 – 51.8
Total	575	229 (39.8)	22.6 – 28.3

95% CI- 95% confidence interval

Table 13 shows the results of univariate logistic regression analysis of predictors/risk factors (age group, gender, previous history of TB, region of residence, location, age group, sex and location) for the primary study outcome. All age groups were three time as likely to have NTM in their sputa as the 15-24 age group, while those who resided in urban areas were 40% less likely to be NTM positive. Women were also more likely to have NTM than men. In the adjusted model, age, sex and location remained significantly associated with NTM

Table 13: Investigating the risk factors associated with pulmonary NTM (n=575)

Risk factor	Unadjusted odds ratio (95% CI)	*p*-values	Adjusted odds ratio (95% CI)	*p*-values
Age group				
15-24	1		1	
25-34	3.77 (1.32 – 10.79)	0.013	3.2 (1.10 – 9.3)	0.034
35-44	3.52 (1.36 – 9.10)	0.009	2.92 (1.11 – 9.10)	0.029
45-54	2.90 (1.14 – 7.40)	0.026	2.33 (0.90 – 6.07)	0.081
55-64	2.4 (0.94 – 6.13)	0.066	2.41 (0.80 – 5.34)	0.133
≥65	3.36 (1.38 – 8.17)	0.007	2.30 (1.21 – 7.37)	0.017
Sex				
Male	1		1	
Female	1.46 (1.02 - 2.09)	0.039	1.53 (1.02 - 2.09)	0.023
Location				
Rural	1		1	
Urban	0.47 (0.26 - .85)	0.014	0.47 (0.26 - 0.84)	0.012
Prior TB				
Yes	1			
No	1.44 (0.69 – 3.01)	0.330		
Participant categories				
No respiratory symptoms; normal CXR	1			
No respiratory symptoms; abnormal CXR	0.76 (0.26 – 2.19)	0.609		
Respiratory symptoms; abnormal CXR	0.67 (0.22 - 1.99)	0.469		

Respiratory symptoms; normal CXR	0.82 (0.28 – 2.40)	0.715

4.8.5 Culture Results and Mycobacterium Species Identification and Distribution

A total of 1,145 stored decontaminated sputa (from 903 suspected cases of NTM) were inoculated in this study. These yielded 56.0% (641/1,145) of instrument positive AFB positive cultures. Mycobacteria specific *16S rRNA* gene PCRs amplified 47.3% (303/641) of AFB positive cultures as mycobacteria. Sequences from 91.7% (278/303) of samples amplified as mycobacteria, were included in the final analysis of which 98.2% (273/278) were identified as NTM while 1.8% (5/278) cultures were MTBC. Nearly a tenth (20/273) of NTM in the study could not be characterised to species level (see Figure16 below).

The phylogenetic tree (Figure 17) constructed using the sequences obtained shows 71.1% (194/273) of all NTM sequences in this study clustered with *M. avium* and *M. intracellulare* (*M. avium* complex) reference strains while 10.0% (26/273) clustered with *M. fortuitum* and *M. boenickei* (*M. fortuitum* complex). Other NTM identified - *M. nonchromogenicum, M. thermoresistible, M. terrae* and *M. flavescens,* were 100% concordant in clustering with respective reference strains. However, 20 NTM sequences could not be identified especially as they did not cluster with any of the NTM and MTBC reference sequences. The five MTBC strains identified in the study also clustered with MTBC reference strains. Table 14 displays the mycobacteria species identified, stratified by the different participant groupings in the present study

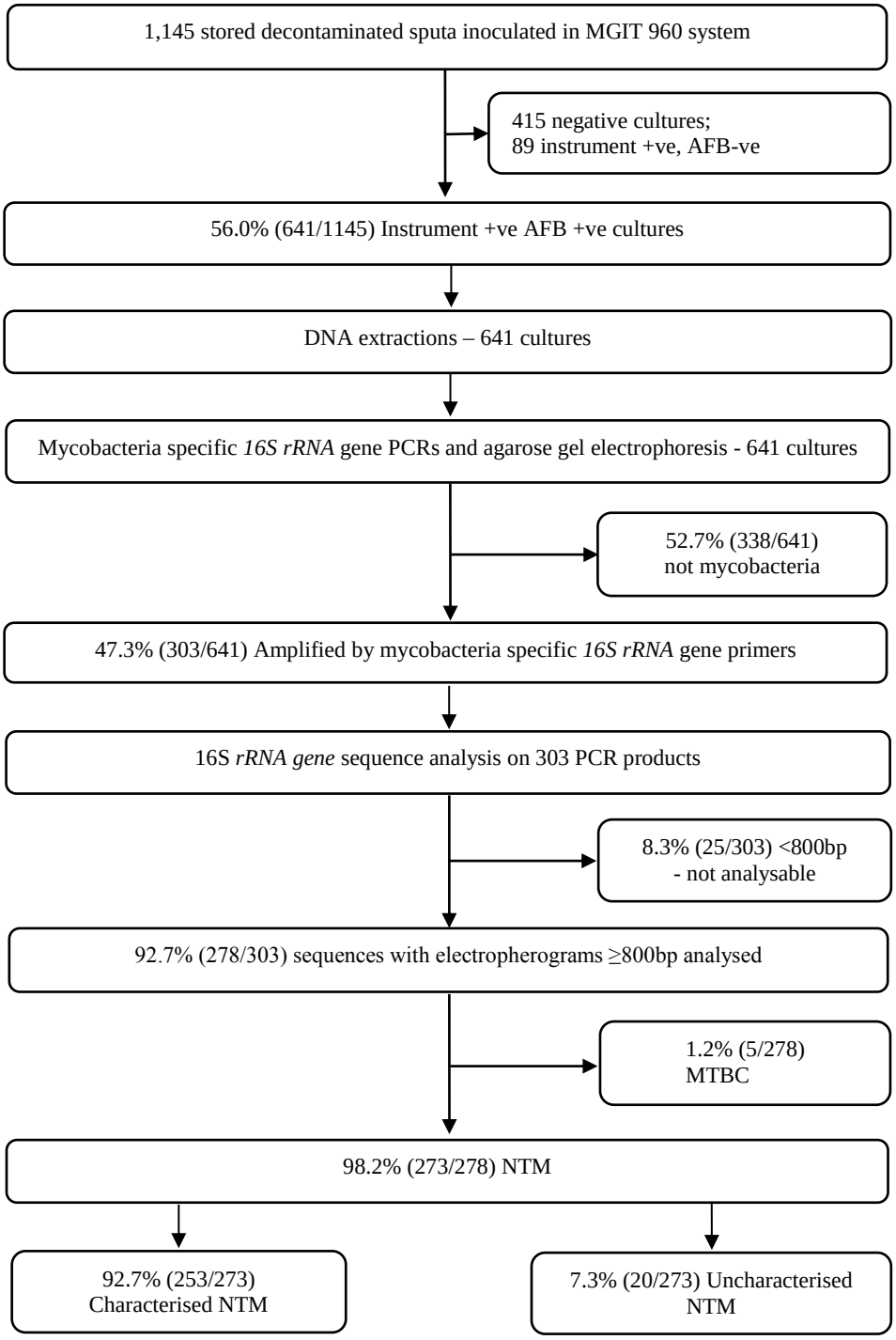

Figure 16: Culture results of non-tuberculous mycobacteria in eligible sputum samples at different stages of sample processing

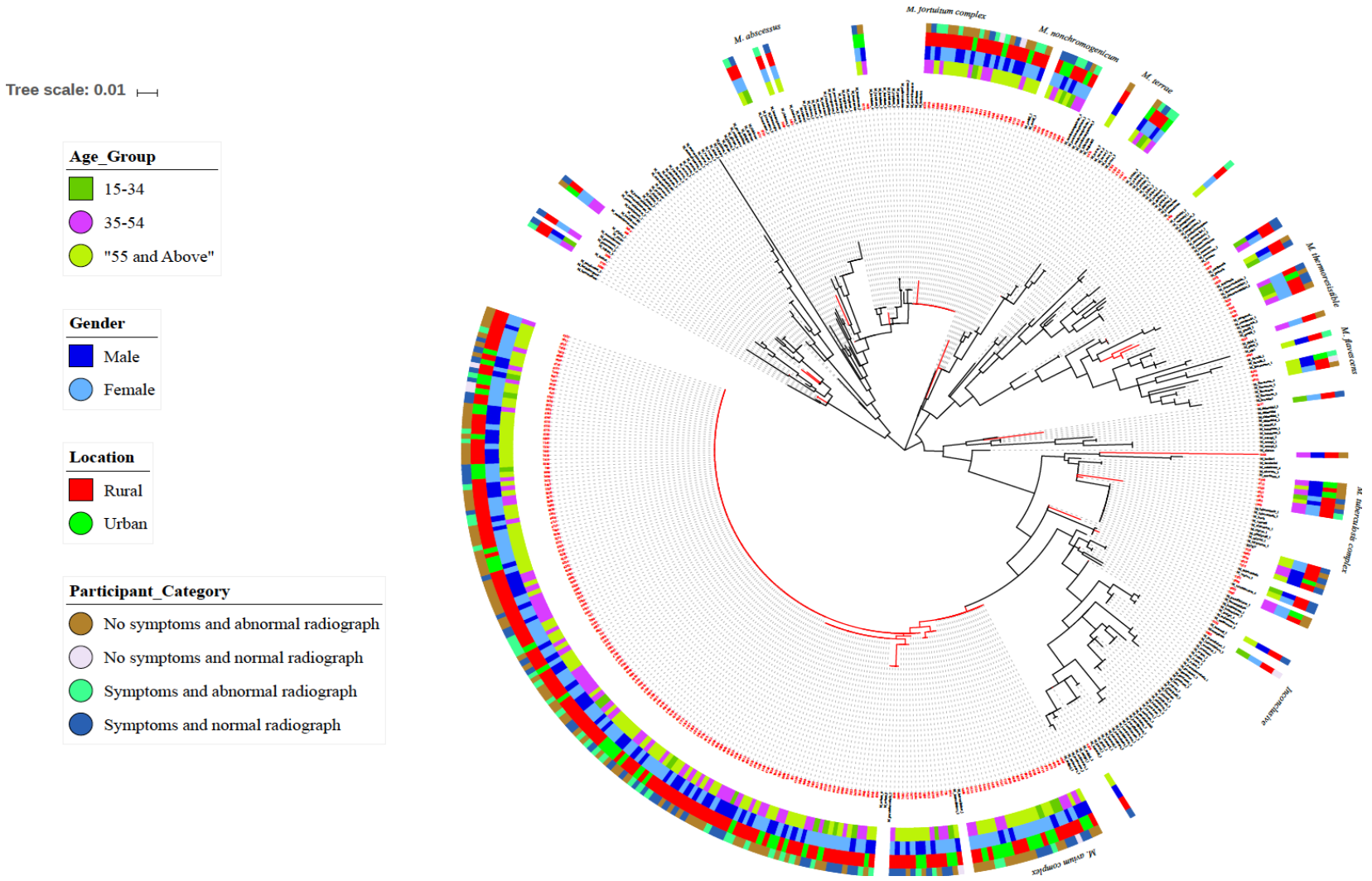

Figure 17: Phylogenetic tree of NTM in The Gambia based on *16S rRNA* gene sequence analysis of regions A and B of mycobacteria gene. Red coloured fonts represent unknown sequences from the present study while fonts in black colour represent NTM and MTBC reference sequences of ATCC strains downloaded from GenBank

Table 14: The distribution of non-tuberculous mycobacteria species in four participant categories

Mycobacteria species	n (%)	No respiratory symptoms; abnormal CXR	No respiratory symptoms; normal CXR	Respiratory symptoms; abnormal CXR	Respiratory symptoms; normal CXR
M. avium complex	194 (71.0)	94	5	41	54
M. fortuitum	26 (9.5)	12	2	8	4
M. nonchromogenicum	8 (2.9)	1		3	4
M. terrae	5 (1.8)	1		2	2
M. thermoresistible	4 (1.5)	1			3
M. abscessus	2 (0.7)			1	1
M. elephantitis	2 (0.7)	2			
M. brumae-like	2 (0.7)				2
M. flavescens	3(1.1)		1	2	
M. duvalii	1 (0.4)			1	
M. goodii	1 (0.4)	1			
M. cosmeticum	1 (0.4)			1	
M. hodleri/isoniacini	1 (0.4)	1			
M. smegmatis	1 (0.4)	1			
M. triviale	1 (0.4)			1	
M. komossense	1 (0.4)				1

Uncharacterised NTM	20 (7.3)	7	1	3	9
Total number of cultures	273 (100)	121	9	63	80

4.8.6 Prevalent pulmonary non-tuberculous mycobacteria disease

The ATS/IDSA diagnostic criteria (see Table 2) was applied to determine the clinical relevance of isolated NTM to the 19.2% of participants (44 of 229) that grew NTM in both sputa. Only 20.5% (9/44) had clinically relevant pulmonary NTM and all were caused by MAC. These nine pateints met the full criteria for pulmonary NTM disease with respiratory symtoms, abnormal CXR and same species of NTM found in duplicate sputum samples (Figure 18). One patient, 2.3% of 44, had *M. fortuitum* in both sputa but did not meet clinical criteria for NTM disease as this patient had respiratory symptoms without an abnormal CXR. All the clinically relevant pulmonary NTM cases belonged to the participant group with respiratory symptoms and CXR abnormalities suggestive of TB (Table 15). Given the challenge with obtaining two sputum samples from participants in the parent study, additional diagnostic categories of confirmed, probable, suspected and non-pulmonary NTM disease are presented in Table 16 below [61, 85].

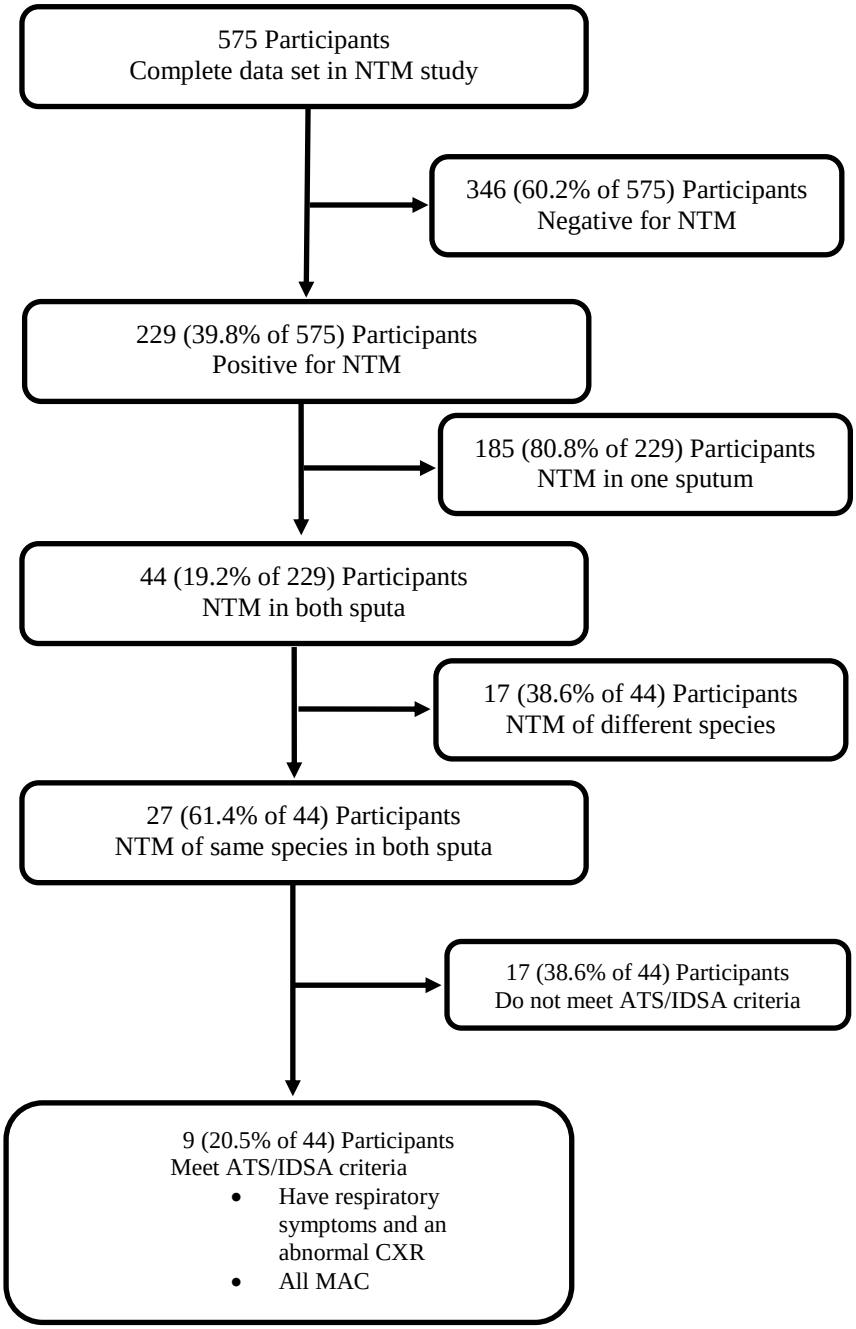

Figure 18: Outline for clinically relevant NTM in participants with NTM in both sputa.

Table 15: The distribution of American Thoracic Society/Infectious Disease Society of America defined clinically relevant pulmonary NTM by participant categories

Participant category	Proportion with NTM in both samples	Proportion with NTM of same species in both samples	Meets ATS/IDSA/ number
No respiratory symptoms; abnormal CXR	19/44 (43.2%)	10/44 (22.7%)	0
No respiratory symptoms; normal CXR	1/44 (2.3%)	0/44 (0)	0
Respiratory symptoms; abnormal CXR	13/44 (29.5%)	9/44 (20.6%)	9
Respiratory symptoms; normal CXR	11/44 (25.0%)	8/44 (18.2%)	0
Total	44/44 (100%)	27/44 (61.4%)	9/44 (20.5%)

***American Thoracic Society and Infectious Disease Society of America Diagnostic Criteria for pulmonary non-tuberculous mycobacteria.**

Table 16: Distribution of patients with confirmed, probable, suspected and non-PNTM disease among in study population, n= 575

Category	Parameters	Numbers (%)
Confirmed pulmonary NTM disease	Patient with NTM of same species in both samples, respiratory symptoms, abnormal CXR	9 (1.6)
Probable NTM disease	Patient with NTM in one sample, respiratory symptoms, abnormal CXR	36 (6.3)
Suspected NTM disease	Patient with different NTM in both sputum samples, respiratory symptoms and abnormal chest CXR	4 (0.6)
Non-pulmonary NTM disease	Patients with NTM in one or both sputum samples with one of the following features: no respiratory symptoms nor abnormal CXR or no respiratory symptoms with abnormal CXR, respiratory symptoms without abnormal CXR	526 (91.5)

4.9 Discussion

In this chapter, I have described the epidemiology and population biology of NTM isolated from pulmonary samples in a representative sample of the Gambian population with respect to carriage and clinical disease. The availability of detailed clinical, radiological and microbiologic data enabled the discrimination of pulmonary NTM carriage from disease through the application of the ATS/IDSA diagnostic criteria. All clinically relevant pulmonary NTM were caused by the same NTM species, MAC, which was the most prevalent species in carriage. This appears to be the first report on the prevalence of pulmonary NTM colonization and disease using a nationally representative sample in a West African country.

This study was executed using the acceptable standards for identification of NTM at every step, from cultures to identification by the *16S rRNA* gene analysis techniques that I optimised for this study [88]. The results are robust and this is further supported by the absence of typical NTM contaminants, a well-documented confounder in other studies where for example, subjects rinsed their mouths with *M. gordonae*-contaminated tap water before submitting sputum [2, 27, 65].

Pulmonary NTM colonisation and disease rates vary globally. In North America and Europe, disease and colonisation in the general population range from approximately 1-15/100,000 persons and 0.1-2/100,000 persons respectively. These rates are largely unknown for many countries in sub-Sahara Africa where there is a high burden of active MTBC disease [132, 138, 139]. In this study, the estimated prevalence of NTM in AFB positive sputum cultures that were negative for MTBC [according to the BD MGIT ^{TM} TBc Identification (Becton, Dickinson and Company, USA) rapid test] and considered suspicious for NTM regardless of

clinical relevance, is 39.8%. The prevalence here is higher compared to reports from other countries in the sub continent i.e. Nigeria, Ghana, Zambia, Mozambique, Mali and Zimabwe [53, 92, 103, 104, 109, 140-142]. However, of all these other studies, only the Zambian and Zimbawean ones were similar in design to this Gambian survey of a nationally representative population sample. Given that there was missing data for up to 36.2% of the Gambian NTM study participants as previously highlighted, NTM prevalence in this study was estimated using a complete case analysis approach. Multiple imputation (MI) analysis could have also been used to produce an NTM prevalence estimate that utilises all of the data for comparison. However, given the complexity of MI techniques, and following statistical advice from my institution, this approach was considered beyond the scope of this MPhil project.

The top three NTM isolated in this study were MAC, *M. fortuitum*, and *M. nonchromogenicum*, with MAC making up ≥ 70.0% of all NTM put together. This is similar to reports by other investigators in North America and European countries [7, 13, 37, 40, 143], Australia [13, 125, 144], Asia [13, 68, 85, 145, 146] and other countries within sub-Saharan Africa [9, 13, 53, 102, 103, 110] where MAC are the predominant NTM species in pulmonary samples. In my Gambian study, *M. fortuitum* was second only to MAC while *M. thermoresistible*, *M. terrae* and *M. flavescens* were infrequently isolated. As seen in Hoefsloot et al [13], rapidly growing NTM (*M. fortuitum*, *M. abscessus*) accounted for a tenth of all NTM in this study. Contrary to the commonly seen scenario in Europe and Eastern Canada [8, 147, 148], there was no *M. malmoense* and *M. xenopi* colonisation and/or disease in this study population.

Distinguishing between members of the MAC family (*M. intracellulare* and *M. avium*) was not complete in this study suggesting some diversity in the regions A and B of the *16S rRNA*

gene of Gambian MAC strains when compared to those isolated in other regions of the world. This hypothesis requires further investigations using molecular techniques of higher discriminatory power such as Whole Genomic Sequencing. The issue of diversity for NTM most likely extends beyond MAC given that there were no *16S rRNA* gene sequences in the National Centre for Biotechnology Information (NCBI) data base for 7.3% of NTM in this study. This meant that I was unable to identify these NTM down to species level. The clinical implications of this group of uncharacterised NTM in The Gambia requires further investigation to determine their contribution to pulmonary colonisation and disease.

Only small proportion of participants, 1.6% had confirmed pulmonary NTM disease based on the ATS/IDSA diagnostic criteria. These diagnostic criteria, despite being widely adopted, have not been validated for evaluation of pulmonary NTM disease in The Gambia and sub-Saharan Africa as a whole. It is possible therefore, that these criteria may be affected by geographical, socioeconomic and other predisposing factors that are distributed differently in this part of the world. Therefore NTM isolated from participants in the other diagnostic categories (probable and suspected PNTM disease) may be clinically relevant. Considering that the probability of subsequent clinical disease with one positive NTM culture following initial investigation is 4-14% and higher in risk groups, these other categories require further investigation [30, 149]. Other guidelines for diagnosing pulmonary NTM diseases include the microbiological (microbiological component of ATS/IDSA) diagnostic criteria [7, 61], and the British Thoracic Society (BTS) guidelines. The latter were first puplished in the year 2000 and a draft of the updated version is available online for public consultation [150]. Compared to the ATS/IDSA diagnostic criteria, the BTS guidelines are not as widely used and reported in literature for differentiating colonisation from disaease.

16S rRNA gene sequence analysis detected a few more MTBC cases that were missed in the prevalence study. This is not surprising given that molecular methods used in this study are more sensitive than phenotype-based ones used in the prevalence survey. In addition, a few studies have reported mutations in the MPT64 antigens (used for confirmation of MTBC cultures) that may have resulted in false MPT64 negative results [151, 152].

Risk factors for pulmonary NTM in sub-Saharan Africa have not been well characterised. HIV is a well-recognised driving force for NTM disease for sub-Saharan Africa given its association with disseminated NTM disease in those with AIDS [39, 118, 143]. Other commonly described risk factors for NTM in industrialised countries include structural lung damage (due to chronic obstructive disease, cystic fibrosis, cumulative exposure to tobacco, prior tuberculosis) and immunosenescence [39, 40]. Although this study did not explore HIV infection as a risk factor for NTM, (because information on HIV status for participants in the parent study was not collected) others have shown NTM are increasingly being isolated among HIV infected cases [10, 53, 103, 119, 153]. For example, in an evaluation of a cohort of 721 persons living with HIV/AIDS in Cote d'Ivoire, the incidence of NTM disaease was 9.7 times higher among patients with baseline CD4 cell counts less than 100 cells/mm^3 compared to those above 100 cells/mm^3 [36]. This finding confirms the significant importance of T-cell immunity in the role of NTM and the role of HIV as a major driver of NTM infection both in industrialised countries (before the introduction of highly active retroviral therapy) and low and middle income countries endemic for TB.

Rural residence in The Gambia was independently associated with NTM disease infection regardless of disease. This may be due to the generally dusty nature of the rural parts of The Gambia which together with poor hygiene and nutrition have been shown to increase the risk

for NTM disease [3, 154, 155]. Higher risks for environmentally acquired pulmonary mycobacterial diseases have also been previously reported among farmers [3] and individuals with occupational exposure to dust [154]. Contrary to the above finding, a review from Oregon reported pulmonary NTM disease to be more associated with urban regions, suggesting a role for urban municipal water supply in NTM transmission and NTM disease [156]. As reported elsewhere [2, 7, 40, 157], female sex was positively associated with NTM in this study. In the case of gender, reports suggest the epidemiology of NTM disease has changed over the last three decades to affect women more frequently than men . The reasons for this sex differential are unknown. One hypothesised pathophysiological pathway is the lower levels of dehydroepiandrosterone - sulphate (DHEA-S) in women with MAC disease compared with control subjects [158]. However, the study had a small sample size (35 females) that did not allow for firm conclusions to be drawn.

There were a few limitations to this study. Since the parent study was primarily designed to determine the burden of bacteriological confirmed TB in The Gambia , it could have been underpowered to determine the prevalence of pulmonary NTM in both disease and colonisation. However, this is probably unlikely given the precision around my estimates. The unavailability of HIV test results or status in the parent study prevented any investigation of HIV as a risk factor for pulmonary NTM colonisation and/or disease. Given HIV prevalence is only 1.8% [1.4% - 2.3%] in The Gambia, the contribution of HIV co-infection is most likely minimal [159]. The clinical relevance of NTM in younger children could not be ascertained as the parent study only collected specimens from participants aged 15 years or more. However, NTM in other parts of the world is predominantly found in older age groups. Finally, the methods used for NTM identification could not fully differentiate the MAC and

some other species isolated in my study due to limited coverage of the *16S rRNA* gene database. WGS techniques with proven sensitivity and higher discriminatory power would have been more useful for differentiating MAC species and for full identification of the NTM in this study.

In conclusion, this report highlights the contribution of NTM to colonisation and pulmonary disease in The Gambia. NTM as commensals in pulmonary samples may confound the diagnosis of pulmonary tuberculosis, especially in those with abnormal chest radiographs. Given the risk of NTM-related misdiagnosis of PTB, it may be useful to review the existing tuberculosis identification methods for presumptive tuberculosis suspects in The Gambia. Additional research and surveillance is therefore required to investigate the full contribution of NTM to pulmonary disease, particularly in high risk groups.

5 Concluding Remarks and Future Pursuits

Collectively, the findings from my research highlight the contribution of NTM to pulmonary colonisation and disease in sub-Saharan Africa.

As described in the systematic review in chapter 2 [Okoi C, et al. submitted], several studies in sub-Saharan Africa have investigated the frequency of pulmonary NTM among presumptive TB cases [53, 104, 108, 110, 140] [Okoi C, et al. submitted], and patients with "chronic" pulmonary TB [10, 102, 160]. Although the above studies did not provide adequate details regarding the proportion of patients who fulfilled criteria for NTM disease, the findings suggest that a substantial proportion of patients in sub-Saharan Africa suspected of pulmonary TB or multidrug resistance TB may actually have pulmonary NTM disease, that is not detected on routine laboratory analysis.

As NTM and MTBC show similar clinical manisfestations, evaluation of the role of NTM has been well neglected until recently, limiting the available epidemiologic data on pulmonary NTM. This further highlights the negative impact of NTM on TB surveillance and control system in the sub continent. Thus, understanding the epidemiology of pulmonary NTM remains crucial to addressing the burgeoning puplic health challenge [7, 8] noted in TB control programs. To this end, a comprehensive multi-country surveillance of NTM is needed to better understand the the extent of the burden of pulmonary NTM in carriage and disease in sub-Saharan Africa, and to design strategic action plans for effective laboratory diagnostics of NTM pulmonary disease and carriage to inform appropriate case management. Finally, given the increasing incidence of pulmonary NTM colonisation and disease registered around the globe, epidemiological and surveillance data should be obtained through the reporting of pulmonary NTM diseases for Public Health decision making.

5.1 Further work

An important follow-up to this work will be to fully characterize the MAC and NTM that were not identified in the study using next generation sequencing techniques. This will inform a more robust and comprehensive data collection on the epidemiology of pulmonary NTM in the Gambia, especially in the area of regional variability within the MAC species.

Mycobcateria specific *16S rRNA* gene PCRs did not amplify 56.3% (324/575) of AFB positive cultures that were negative for the MPT64 rapid antigen test in this study. Given some other organisms belonging to the genus *Nocardia/Rhodococcus* have the same acid and alcohol fast appearance as NTM and MTBC on microscopy and could have been missed by the mycobacteria specific primer PCRs, subjecting this group of samples to standard *16S rRNA* gene PCR may provide additional information on the pathogen profile of pulmonary tuberculosis-like diseases in The Gambia. Pulmonary nocardiosis is often misdiagnosed and treated as TB, sometimes with fatal consequences.

An area of interest around NTM, and one which is under evaluated, is the role of virulence factors. Determining this may aid in deciphering the clinical relevance of isolated NTM from clinical samples. The species *M. kansasii* provides a good example: in a previous case series, *M. kansasii* subtype 1 was most strongly associated with clinical disease while subtypes 3-5 seemed non-pathogenic. A recent study of the ESX-1 virulence factors of *M. tuberculosis* also evaluated their role in *M. kansasii* strains and found that *M. kansasii* sub type 1 had an active ESX-1 system which was inactive in *M. kansasii* sub-type 5 [1, 161]. Assessing such virulence factors could be a valuable addition to species identification.

Finally, a key issue for diagnosing pulmonary NTM is the recognition of patients at increased risk for this disease. With the exception of HIV disease, other factors remain poorly understood especially for patients in sub-Saharan Africa.

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Appendix

Appendix I: MRCG Scientific Coordinating Committee Aproval

Scientific Coordinating Committee
MRC Unit: The Gambia, Fajara
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MRC The Gambia Unit

14 March 2014

Mrs Catherine Okoi
Vaccinology Theme Leader
MRC Unit, The Gambia
Fajara

Dear Mrs Okoi

SCC 1371v2, Non-Tuberculosis Mycobacteria (NTM) in The Gambia: prevalent species, carriage and disease

Thank you for submitting your response letter and revised proposal dated 6 March 10 March 2014 respectively addressing the issues raised by the SCC at its meeting held on 3 March 2014.

I am happy to approve your project proposal. Please note that the implementation of this project would depend on the availability of funds.

Your proposal will be forwarded to the Ethics Committee for consideration at its meeting held on 28 March 2014.

With best wishes

Yours sincerely

Professor Umberto D'Alessandro
Chairman, Scientific Coordinating Committee

Cc: Dr Jayne Sutherland

Additional documents submitted for review:-

- Informed Consent Document (SCC 1232), Version 2.0 – 17 February 2010
- Ethics reply letter re SCC 1232 – 30 May 2011

Appendix II: Gambia Government/MRCG Joint Ethics Committee Approval

The Gambia Government/MRC Joint
ETHICS COMMITTEE

C/o MRC Unit: The Gambia, Fajara
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31 March 2014

Ms Catherine Okoi
Vaccinology Theme
MRC Unit, The Gambia
Fajara

Dear Ms Okoi

SCC 1371v2, Non-Tuberculosis Mycobacteria (NTM) in The Gambia: prevalent species, carriage and disease

Thank you for submitting your proposal dated 10 March 2014 for consideration by the Gambia Government/MRC Joint Ethics Committee at its meeting held on 28 March 2014.

We are pleased to approve your proposed study.

With best wishes

Yours sincerely

Mr Malamin Sonko
Chairman, Gambia Government/MRC Joint Ethics Committee

Documents submitted for review:-

- SCC Application form, version 2.0 – 10 March 2014
- SCC approval letter dated 14 March 2014
- Informed Consent Document (SCC 1232), Version 2.0 – 17 February 2010
- Ethics reply letter re SCC 1232 – 30 May 2011

Appendix III: Participant information sheet and consent form for the Gambia Survey of TB prevalence study

National Leprosy and Tuberculosis Programme
Ministry of Health and Social Welfare

The Gambian Survey of TB Prevalence (GAMSTEP) SCC 1232
Subject Information Sheet and Consent Form-Form 3

MRC The Gambia Unit

The Gambian government through the Ministry of Health and Social Welfare and the National Leprosy and Tuberculosis Programme wants to know how badly Tuberculosis (TB) affects Gambians. This is important because despite government's efforts to control TB an increasing number of cases are still being found every year. In order to do this, the National TB Programme has entered into partnership with the Medical Research Council (UK) Unit, The Gambia to help conduct this survey. Information from this survey will provide government and partners with better understanding of the burden of TB in The Gambia so they can plan and implement TB prevention and control activities better.

TB is an illness that is caused by a germ and is transmitted via the airborne route i.e. inhaled when people with active disease cough into the air. It mainly affects the lungs, but sometimes can affect other parts of the body. It is important for you to know that TB is curable and free treatment is available in The Gambia. You have been asked to come here or are visited today because your district, settlement and compound have been selected to participate in this nationwide survey of TB disease. As a resident in this area aged ≥15 years, you are eligible to participate and this is an invitation for you to consent to become a part of this study. If you agree to participate, you will become one of over 55,000 Gambians countrywide expected to participate in this survey.

If you agree to participate in this survey, you will

- Be asked to sign a document agreeing to your participation in the study.
- Participate in a short interview and answer some questions about signs and symptoms related to TB disease.
- Have a chest x-ray done.
- Be asked more questions about your understanding, knowledge, beliefs and other experiences of/with TB.
- Depending on the result of your interview and chest x-ray examination be asked to give a sputum sample two times- twice today or one time today and again tomorrow morning. A team member will explain how you should do this. Asking you for a sputum sample does not mean you have TB. Your sputum sample will be transported to the MRC TB laboratory where it will be examined for the presence of TB.
- Be referred to the nearest health centre for professional advice (where possible), if we think you need other care based on your answers to our questions and/or chest X-ray.
- Will be told by a member of the survey team, if there is any evidence of TB disease found after your chest X-ray and sputum samples have been checked again to confirm or exclude TB disease by clinical, laboratory and radiology experts after today's procedures. The survey team or health care worker closest to you will help ensure you receive proper TB treatment free of charge.
- The CXR images from this survey maybe shared with partners outside of The Gambia as part of efforts to optimise identification of Tuberculosis from CXR obtained with digital technology. If this happens, no personal information will be attached to the images to ensure confidentiality.

While you will not receive any money for your participation, there is benefit if you or those around you or in your community are found to have TB since prompt diagnosis and referral for treatment will reduce the duration of illness and prevent transmission of the disease to close contacts. You are not exposed to any risk by participating in this survey. Some people have genuine concerns about X-rays. We assure you that X-rays are safe when used properly. The MRC has significant experience in the use of X-ray with no history of any problems for over 20 years. The Radiographers and x-ray technologists have been trained to use the minimum amount of radiation necessary to obtain the needed results without any risk on your health. In addition, the mobile digital X-rays are associated with very little risk of radiation exposure. If you are pregnant, please let the radiology team know as they can take your chest X-ray with a protective lead jacket over your abdomen. If you have any additional concerns, we can provide more information to you.

National Leprosy and Tuberculosis Programme
Ministry of Health and Social Welfare

The Gambian Survey of TB Prevalence (GAMSTEP) SCC 1232

Subject Information Sheet and Consent Form-Form 3

MRC The Gambia Unit

Please NOTE your participation is voluntary, you are not being coerced to participate in this study. If you feel that way please contact any of the contact persons with address on this form. You can refuse to participate without any penalty or loss of benefits to which you are normally entitled to. You can withdraw your consent/participation at any time and your refusal will have no effect on your current or future relationships with the survey team or institutions conducting this study.

The Government of the Gambia is aware of this survey and is a partner in its conduct through the National Leprosy and Tuberculosis Control Programme. The Governor/Mayor and the regional health team are also aware of this survey as well as your alkalo, village development committee, community health nurse and village health worker. They can confirm or refute what you have been told by the survey staff.

We know you may have concerns about your personal details including medical history related to TB becoming known to others. Please note the records of this study will be stored securely and kept confidential. All publications and/or reports that may arise from this survey will not include any information that will make it possible for you to be identified. However, if you are found to have TB, we will refer you to the National TB Programme at the nearest chest clinic for treatment or additional investigations where required. This referral will include your name and other personal identifiers which are required to get you on treatment which is to your benefit.

You can ask as many questions as you want to about this survey or TB in general. The MRC staff discussing this study with you can answer your questions and if you want additional information or questions to ask later, please contact the survey coordinator who is around in your community now or the project administrator and/or principal investigator whose contact details are listed on this form

Contact details:

Project administrator: Mrs. Elina Cole, 4495442-6, ext 3012, 3813925

Survey Coordinator: Mr. Ma Ansu Kinteh, 4495442-6, ext 3013, 9931197

Principal Investigator: Dr. Ifedayo Adetifa, 4495442-6, ext 3011, 3820780

Appendix

National Leprosy and Tuberculosis Programme Ministry of Health and Social Welfare

The Gambian Survey of TB Prevalence (GAMSTEP) SCC 1232
Subject Information Sheet and Consent Form-Form 3

MRC The Gambia Unit

The Gambian Nationwide Tuberculosis Prevalence Survey SCC 1232
CONSENT CHECKLIST

Subject's name: _____

Individual Survey No. ☐☐☐ ☐☐☐ ☐☐☐ Cluster Nos |__|__|

Cluster Name_____

1. I have understood the information sheet and I have had the chance to ask questions about the study.
   Yes ☐ No ☐
2. I will be asked questions related to signs and symptoms of TB and may be asked additional questions about my understanding, knowledge, beliefs and other experiences of/with TB **Yes ☐ No☐**
3. I will be asked to have a chest X-ray at no cost to me. **Yes ☐ No☐**
4. Depending on the answer I give to the questions asked about TB symptoms and findings on my chest X-ray, I will be asked to produce 2 or more sputum samples to be tested for the presence of the germ that causes TB.
   Yes ☐ No ☐
5. I understand my chest X-ray and sputum samples will be checked to confirm or exclude TB disease.
   Yes ☐ No ☐
6. If there is any evidence of TB disease found, I will be told by a member of the survey team or health care worker closest to me who will ensure I receive proper treatment free of charge.
   Yes ☐ No ☐
7. My medical information will remain confidential and will be used only for the purposes of the study.
   Yes ☐ No ☐
8. I understand that part of my sputum may be stored for possible future tests.
   Yes ☐ No ☐
9. I understand that I do not have to take part in this study, and that I can leave the study at any time and without giving any reason. **Yes ☐ No ☐**

Signature (or thumb-print) of volunteer: ..... Date ...../...../.....
For minors (15-17 years), signature (or thumb-print) of guardian or parent: .....
Date...../...../.....

I have read the above to :.....(PRINT NAME OF VOLUNTEER) in a language, which he/she understands. I believe that he/she gives consent to take part in the study.

Signature of Field Worker:..... Name:..... Date ...../...../.....

2. Risk Factors

2.1. Have you ever smoked.....|__| (1 = Yes; 2=No; 3=-yes, but stopped less than 6 months ago; 4 = yes, but stopped more than 6 months ago) **If 'NO', go to question 2.4**

2.2. Frequency of smoking |__| (1 = not every day; 2 = 1-5 times/day; 3 = 6-10/day; 4 = 10/day; 5=not applicable)

2.3. Duration of smoking |__| (1 = less than one year; 2 = 1-5 years; 3 = 5-10 years; 4 = >10 years; 5=not applicable)

2.4. Do you drink Alcohol |__| (1 = Yes; 2=No; 3=-yes, but stopped less than 6 months ago; 4 = yes, but stopped more than 6 months ago) **If 'NO', go to question 3**

2.5. Duration of drinking |__| (1 = less than one year; 2 = 1-5 years; 3 = 5-10 years; 4 = >10 years; 5=not applicable)

2.6. How often do you drink? |__| (1=occasionally, 2=1-2 times/week; 3=3-5 times/week; 4=>5 times/week; 5=not applicable)

3. For females only: As you will have a chest X-ray is done it is important we know if you think it is possible you are pregnant |__| (fill 0=No, 1=Yes, 2=Not sure)

4. Eligibility

4.1. Are symptoms eligible for sputum examination (as per criteria in Q1)? |__| (fill 0=No, 1=Yes)

4.2. Is participant eligible for re-interview? |__| (fill 0=No, 1=Yes) **FILL Yes, if answer to 4.1 is YES**

5. **Chest X-Ray (TO BE FILLED BY SURVEY CLINICIAN)**

5.1. **Chest X-ray** |__| (fill 1=Yes, 2=No,3=Exempted, reason _____)

5.2. **Date X-ray taken** |__|__|/ |__|__|/ |__|__|__|__|
Day month Year

5.3. **Chest X-Ray result by field screening**

5.3.1.Clinician/X-ray Reader |__| (Fill 0=Normal, 1=Abnormal for TB, 2=Other abnormality, not TB, 3=unsure)
IF UNSURE USE COMPUTER AIDED CXR INTERPRETATION

5.3.2.Computer aided X-ray reader |__| (Fill 0=Normal, 1=Abnormal, 2=Other abnormality, not TB 3=Other)
specify_____

5.4. **Eligibility**

5.4.1.Is subject eligible for sputum collection based on CXR|__| (fill 1=yes, 2=no)

5.4.2.Is subject eligible for re-interview|__| (fill 1=yes, 2=no) **FILL Yes, if answer to 5.4.1 is YES**

REFER TO DATA CHECKER

TO BE FILLED IN AT DATA CHECKER STATION

6. Eligibility

6.1 Is subject eligible for sputum collection|__| (fill 1=yes, 2=no)
IF YES, REFER TO SAMPLE COLLECTION STATION

IF YES, REFER TO FIELD SUPERVISOR FOR RE-INTERVIEW

TO BE FILLED IN AT SAMPLE COLLECTION STATION

7. Dates of sample collection

7.1 SP1 collection date|__|__|/ |__|__|/ |__|__|__|__|
Day month Year

7.2 SP2 collection date |__|__|/ |__|__|/ |__|__|__|__|
Day month Year

END OF INTERVIEW

2

Appendix IV: Participant information sheet and consent form for the Gambia Survey of TB prevalence study

National Leprosy and Tuberculosis Programme Ministry of Health and Social Welfare

The Gambian Survey of TB Prevalence (GAMSTEP) SCC 1232

Tuberculosis Screening Questionnaire-Form 4

MRC The Gambia Unit

Decisions checklist to be filled in after completing each portion of the form

1. KAP done ☐ Yes ☐ No
2. Reason for sputum sample ☐ Has TB symptoms ☐ Has abnormal CXR ☐Exempted from CXR ☐ Not eligible to give sputum
3. Reason for re-interview ☐ TB Symptoms ☐ Has abnormal CXR ☐ Not re-interviewed

Individual Survey No. ☐☐☐ ☐☐☐ ☐☐☐

(To be filled in by receptionist)

Date |__|__|/ |__|__|/ |__|__|__|__| **Interviewers/FW code:** |__|__|

Day month Year

1. **Name:** _____
2. **Age:** |__|__|years 3. **Sex:** ☐ Male ☐ Female 4. **Cluster Name**_____
5. **Marital status** |__| (1.Single 2.Married 3.Separated/divorced 4.Widow/Widower)
6. **Ethnic group** |__| (1=Mandinka,2=Wolof,3=Fula,4= Serahule,5=Jola,6=Aku,7=Serere,8= Manjago, 9=other, specify_____)
7. **Education** |__| (1.Illiterate 2. can read/write in English or Arabic 3.Primary school 4.Incomplete Secondary school 5.Secondary School 6.Diploma/equivalent 7.University Graduate 8. Post graduate (Masters, Post graduate diploma, PhD))
8. **Occupation** |__| (1.Professional, technical and worker 2.Own Business 3. Merchant 4. Service worker (including Government servant) 5. Trader 6. Agricultural, animal husbandry and forestry worker, fisherman and hunter 7.Production and related worker, transport, equipment operator and labourers 8.House wife 10.student 11.Dependent 12. Clergy 13. Others (please specify) _____)

1. **Medical History (ask about the presence of these symptoms at this current time):**
   - 1.1. Does the participant have a cough? |__|**(fill in 0=No, 1=Yes) If No, GO TO Q 1.3**
   - 1.2. How long has the participant been coughing? |__| **(1=cough <1 week, 2=cough 1-2 weeks,3=Cough ≥2 weeks, 9=unknown)**
   - 1.3. Does the subject have **(Fill 1=yes, 2=no for the following)?**
     - 1.3.1.Weight loss |__|
     - 1.3.2.Fever |__|
     - 1.3.3.Drenching night sweats |__|
     - 1.3.4.Chest pain |__|
     - 1.3.5.Shortness of breath |__|
     - 1.3.6.Loss of appetite |__|
     - 1.3.7.Haemoptysis (Coughing up blood) |__|
   - 1.4. Have you ever been treated for tuberculosis before? |__| **(fill 1=yes, 2=no)**
   - 1.5. Are you currently on TB treatment |__| **(fill 1=yes, 2=no), if yes, GO TO 1.6**
   - 1.6. When did you start TB treatment? |__|__|/ |__|__| month Year

NOTE: Only those with cough for 2 weeks or more from Q1.2 OR those with cough for <2 weeks AND 2 or more symptoms from Q1.3 OR 3 or more symptoms from Q1.3 are eligible for sputum examination.

Ver 3.0 23/11/11

Appendix V: Full drafts of publications

Research

A tuberculosis nationwide prevalence survey in Gambia, 2012

Ifedayo MO Adetifa,[a] Lindsay Kendall,[b] Adedapo Bashorun,[b] Christopher Linda,[b] Semeeh Omoleke,[b] David Jeffries,[b] Rahmatulai Maane,[c] Beatrice Dei Alorse,[c] William Dei Alorse,[d] Catherine Bi Okoi,[d] Kodjovi D Mlaga,[d] Ma Ansu Kinteh,[b] Simon Donkor,[b] Bouke C de Jong,[e] Martin Antonio[d] & Umberto d'Alessandro[b]

Objective To estimate the population prevalence of active pulmonary tuberculosis in Gambia.
Methods Between December 2011 and January 2013, people aged ≥ 15 years participating in a nationwide, multistage cluster survey were screened for active pulmonary tuberculosis with chest radiography and for tuberculosis symptoms. For diagnostic confirmation, sputum samples were collected from those whose screening were positive and subjected to fluorescence microscopy and liquid tuberculosis cultures. Multiple imputation and inverse probability weighting were used to estimate tuberculosis prevalence.
Findings Of 100 678 people enumerated, 55 832 were eligible to participate and 43 100 (77.2%) of those participated. A majority of participants (42 942; 99.6%) were successfully screened for symptoms and by chest X-ray. Only 5948 (13.8%) were eligible for sputum examination, yielding 43 bacteriologically confirmed, 28 definite smear-positive and six probable smear-positive tuberculosis cases. Chest X-ray identified more tuberculosis cases (58/69) than did symptoms alone (43/71). The estimated prevalence of smear-positive and bacteriologically confirmed pulmonary tuberculosis were 90 (95% confidence interval, CI: 53–127) and 212 (95% CI: 152–272) per 100 000 population, respectively. Tuberculosis prevalence was higher in males (333; 95% CI: 233–433) and in the 35–54 year age group (355; 95% CI: 219–490).
Conclusion The burden of tuberculosis remains high in Gambia but lower than earlier estimates of 490 per 100 000 population in 2010. Less than half of all cases would have been identified based on smear microscopy results alone. Successful control efforts will require interventions targeting men, increased access to radiography and more accurate, rapid diagnostic tests.

Abstracts in عربي, 中文, Français, Русский and Español at the end of each article.

Introduction

Tuberculosis killed 1.5 million people in 2014 and is the leading cause of death from an infectious disease worldwide.[1] Sub-Saharan Africa, with 28% (9.6 million) of all notified tuberculosis cases in 2014, endures a disproportionate burden of the disease relative to its population. In Gambia, the estimated incidence and prevalence of tuberculosis rose from 258 and 350 per 100 000 population respectively in 1990 to 284 and 490 per 100 000 in 2011.[2] In addition, the tuberculosis case detection rate – that is, the ratio of the number of notified tuberculosis cases to the number of incident tuberculosis cases in a given year – remained low at 48% (95% confidence interval, CI: 40–58).[2] It is not clear if poor case detection is due to inequitable access to care or inadequate diagnosis of tuberculosis in urban or remote parts of the country.

Given the need for improved, evidence-based interventions in tuberculosis control in Gambia, it is important to establish reliable baseline estimates of tuberculosis prevalence against which future control interventions can be assessed. This study therefore aimed to estimate the population prevalence of active pulmonary tuberculosis disease in Gambia, in 2012 and to compare the case detection rate with global tuberculosis control targets.

Methods

Study design

We carried out a nationwide, multistage cluster survey in 2011–2013. A sample size of 55 281 participants ≥ 15 years old from 80 clusters was calculated assuming a prevalence of 292 sputum smear-positive cases per 100 000 population,[3] 85% participation target, design effect of 1.51 and application of a finite population correction.[4] A sample size with 80 clusters was expected to give around 20% precision and higher than 25% precision under the most plausible scenarios, and with an intracluster coefficient of variation of 0.5 the calculated design effect was 1.51.

Sampling to select survey areas was multistage and without any stratification. First, we allocated 80 survey enumeration areas by regions of the country in proportion to population size based on the national 2003 census (Central Statistics Department, Government of Gambia). Following this allocation, the West Coast region with about 28.7% (389 274) of the population was to contribute 23 enumeration areas and the least populated Lower River region contributed four enumeration areas for 5.3% (72 184) of the population. This procedure was similar in outcome to the recommended sampling in proportion to population size.[4] Then we randomly selected the survey enumeration areas (e.g. 23 for the West Coast region) up to a total of 80 for the entire country. Each selected survey enumeration area was paired with between one and two adjacent enumeration areas in whole or part until an adult population of 500–700 was attained.

People eligible for participation were all those aged ≥ 15 years; permanent residents who spent at least one night in the household in the preceding 4 weeks; and visitors who had arrived in the household 4 weeks or more before.

Study procedures

Three teams – each consisting of a research clinician who led a team of 7 trained fieldworkers and a radiographer – performed the fieldwork from December 2011 to January 2013. Data collection in each cluster was done over a seven-day period. The field

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workers, in collaboration with community-selected liaisons, enumerated the population in each cluster 4–6 weeks before actual data collection. The field workers visited the enumerated households to obtain data on household composition and residency, and at the same time discussed the purpose of the survey. For families that were not at home, the team were informed at which time they could return to meet the household members. Community entry meetings with the local chief and other community leaders were also held to provide further information.

The field operation sites were selected based on input from community members to ensure that location was as central as possible to facilitate access by pedestrian travel. Project vehicles were available for eligible disabled participants to bring them to the site.

Screening of eligible participants followed the World Health Organization (WHO) recommended algorithm for tuberculosis prevalence surveys.[4] At the field operation site, participants gave written informed consent before they were interviewed for tuberculosis symptoms (cough, weight loss, fever, drenching night sweats, chest pain, shortness of breath, loss of appetite and coughing up of blood). Then they had chest X-rays taken by a radiographer who used a mobile direct digital radiography machine. Chronic cough lasting two weeks or more is the cardinal symptom in the national tuberculosis guidelines. Participants who screened positive for symptoms and/or had an X-ray suggestive of tuberculosis were asked to submit two sputum specimens. All those unable to have chest X-rays were asked to submit two sputum samples whether they had symptoms or not. Participants submitted the sputum samples at the field site, with a 30–45 minute interval. Participants who were unable to submit samples on-site were given two prelabelled containers for collection early the following morning. All survey participants were given a token gift of laundry soap.

Case ascertainment

Survey clinicians were encouraged to over-interpret X-rays to increase the sensitivity of screening, as recommended.[4] The radiology panel, which included those who trained the X-ray readers, reviewed all abnormal X-rays and 10% of normal films for quality assurance and definitions. The final radiological diagnosis was determined via consensus by a pulmonologist and radiologist.

Sputum specimens collected in the field were stored in a temperature-monitored cold box. If the samples could not be transported to the laboratory that day, the samples were stored in a fridge kept at 4 C in the team's camp. In general samples were transported every 48 hours after sputum collection to the main laboratory, the maximum delay in transportation was 72 hours. Sputum samples were processed at the Tuberculosis Diagnostic and Research Laboratory of the Medical Research Council Unit The Gambia. The laboratory holds good clinical laboratory practice[5] accreditation and subscribes to an external quality assessment service.[6] Sputum smears were examined using fluorescence microscopy. Cultures were performed using the BACTEC Mycobacterial Growth Indicator Tube (MGIT) system (Becton Dickinson, Franklin Lakes, United States of America). Standard laboratory procedures[7] were followed for confirmation of growth in liquid culture including rapid species identification with an immunochromatographic assay (MGIT TBc Identification Test, Becton Dickinson, Franklin Lakes, USA). All acid-fast bacillus (AFB) isolates obtained from cultures were classified as either *Mycobacterium tuberculosis* or nontuberculous mycobacteria.

The final survey case classification, as defined in Box 1, was done by a central panel consisting of infectious diseases and chest physicians, epidemiologists, laboratory experts and tuberculosis programme representatives.[4]

Box 1. **Case definitions for the national tuberculosis prevalence survey, Gambia, 2012**

Laboratory case definitions

Culture-confirmed tuberculosis case: isolation of *Mycobacterium tuberculosis* complex from a sputum specimen.

Sputum smear-positive tuberculosis case: acid-fast bacillus (AFB)-positive by sputum smear examination, i.e. at least one AFB in 100 immersion fields.

Definite survey case

Bacteriologically confirmed tuberculosis case: one positive tuberculosis culture and at least one of the following:

- another sample culture-positive;
- sputum smear-positive; or
- chest X-ray abnormalities suggestive of tuberculosis by central audited reading.

Sputum smear-positive tuberculosis case: one AFB-positive sample and a culture-positive sample.

Probable smear-positive tuberculosis case

An AFB-positive sample and at least one of the following:

- AFB-positive in another sample but not culture-positive, and no isolation of nontuberculous mycobacteria; or
- Chest X-ray abnormal at central reading but not culture-positive, and no isolation of nontuberculous mycobacteria.

Data analysis

There was double entry of survey data in a MySQL version 5.6.19 (Oracle, Redwood Shores, USA) relational database. All data analyses were done using Stata version 12.1 (Stata Corp., College Station, USA). We tested for differences in proportions using two-sample tests of equality of proportions, and conducted multiple imputation to correct for missing data.[8,9] For prevalence estimates, we used three modelling approaches with robust standard errors, missing value imputation and inverse probability weighting applied.[4,8] With multiple runs of chained imputation data sets, trends for the mean values of the four imputed variables (chest X-ray-positive, AFB-positive, culture-positive and bacteriologically confirmed tuberculosis) were obtained for all iterations. We derived subject-level missing values from the relevant combinations of the imputed variables and stratified all prevalence data by sex, age group and residence (rural/urban). Overall prevalence for all forms of tuberculosis – that is, pulmonary and extra-pulmonary – across all age groups were obtained by calculating pulmonary tuberculosis cases (all ages) as a weighted average of tuberculosis in survey participants and in children (obtained from routine reports, Gambian National Tuberculosis and Leprosy Programme). The value obtained was then revised upwards by the proportion of

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all notified tuberculosis cases that were the extrapulmonary type. Incidence was calculated based on assumptions from a set of statistical distributions as described elsewhere.[2]

Ethics approval

The study obtained ethics approval from the joint ethics committee of the Gambian Government and Medical Research Council. All tuberculosis patients identified during the survey were promptly referred to the nearest treatment centre for treatment at no cost and notified to the national tuberculosis programme.

Results

Participants

Enumeration yielded 100 678 people in 13 847 households across 80 clusters, of whom 55 832 (55.5%) were eligible to participate in the survey. The enumerated population was representative of Gambia's population structure (2003 national census) by age and sex (Fig. 1). Of the 55 832 people invited, 43 100 (77.2%) consented to participate. As shown in Fig. 2, there was a significantly higher proportion of female (84.9%; 25 596/30 153) than male participants (68.2%; 17 504/25 679; $P < 0.0001$). In addition, participation was higher in rural (82.3%; 25 554/31 043) than urban (70.8%; 17 546/24 789) clusters ($P < 0.0001$). Overall, participation was slightly less than the 85% target due to lower participation in urban areas and among males. The median age of participants was 28 years (interquartile range: 20–41 years).

Suspected cases

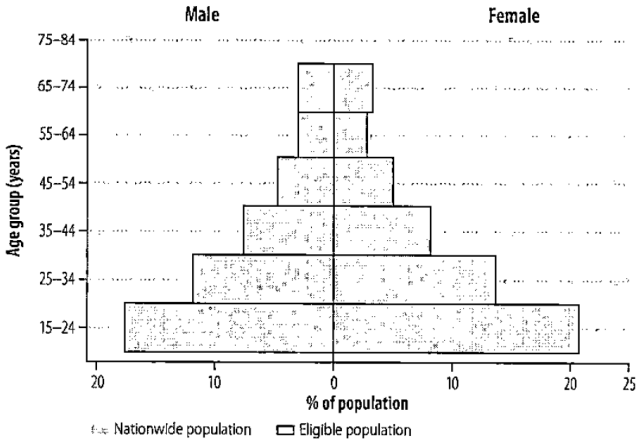
All participants were successfully screened for symptoms and 42 942 (99.6%) by X-ray (Fig. 3); the majority of missing chest X-rays (145/158, 91.8%) were due to patient refusal or ill health, while the remaining (13/158, 8.2%) were due to technical problems.

Cough was reported by 4802 (11.1%) participants. Of those participants knowing the duration of their cough, 962 (2.2%) had had a cough lasting ≥ 2 weeks, 2979 (6.9%) had coughed for < 1 week and 799 (1.9%) had coughed for 1–2 weeks. With respect to other symptoms in the guidelines, 6637 (15.4%) participants reported fever, 6551 (15.2%) chest pain, 1595 (3.7%) night sweats, 2672 (6.2%) shortness of breath, 3620 (8.4%) anorexia and 8448 (19.6%) weight loss. Using other symptom categories, 1372 (3.2%) participants reported cough of < 2 weeks plus two or more of the other symptoms, while 1128 (2.6%) did not have a cough but had three or more other symptoms.

Based on field X-ray screening, 3407 (7.9%) participants had chest abnormalities suggestive of tuberculosis.

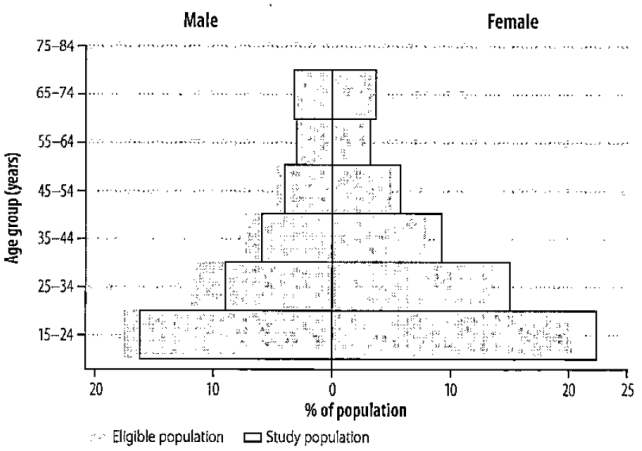
A total of 5948 (13.8%) participants were classified as tuberculosis suspects and were eligible for sputum examination; 2447 were eligible by symptom

Fig. 1. **Population pyramid showing representativeness of eligible population in the national tuberculosis prevalence survey, Gambia, 2012**



Note: Nationwide population was 1 306 681 and eligible population was 55 832 people.
Data source: Gambia 2003 National Census, Central Statistics Department, Government of the Gambia

Fig. 2. **Population pyramid showing representativeness of study population in the national tuberculosis prevalence survey, Gambia, 2012**



Note: Eligible population was 55 832 people and study population was 43 100 people.
Data source: Gambia 2003 National Census, Central Statistics Department, Government of the Gambia

Appendix

screening only, 2382 by chest X-ray screening only and 1050 by both screening methods (Fig. 3).

Definite cases

Laboratory results were available for 5519 participants with suspected tuberculosis. Overall, 77 participants with pulmonary tuberculosis were identified, 71 of which were bacteriologically confirmed. The expert panel categorized 28 participants' diagnostic results as definite, smear-positive (smear-positive, culture-positive), 43 as definite, bacteriologically confirmed (smear-negative, culture-positive) and six as probable, smear-positive (smear-positive, culture-negative; Fig. 3). Of the samples that were both smear positive and culture positive; 34 samples were identified by culture as *M. tuberculosis* and 2 (5.6%) as nontuberculous mycobacteria.

If sputum smear microscopy had been done only in symptomatic people, diagnosis would have been made in 52.9% (18/34) of prevalent smear-positive cases, while chest X-ray abnormalities would have identified 94.1% (32/34). Among all participants with bacteriologically confirmed tuberculosis, symptoms alone identified significantly fewer cases than chest X-ray screening in the field, 60.6% (43/71) versus 84.1% (58/69; $P<0.01$), respectively. In addition, 25/77 patients (32.5%) reported cough for ≥ 2 weeks, 14 (18.2%) for < 2 weeks with two or more symptoms, while 5 (6.5%) did not have a cough but had three other symptoms. In total, 24.7% (19/77) of the participants were identified as having tuberculosis because the patient screened positive for additional symptoms.

Tuberculosis was identified predominantly among male participants (51; 66.2%). The 15–34, 35–54 and ≥ 55 year age groups had 37.7% (29), 39.0% (30) and 23.4% (18) of cases respectively. The numbers of tuberculosis cases were similar in urban (39) and rural (38) clusters.

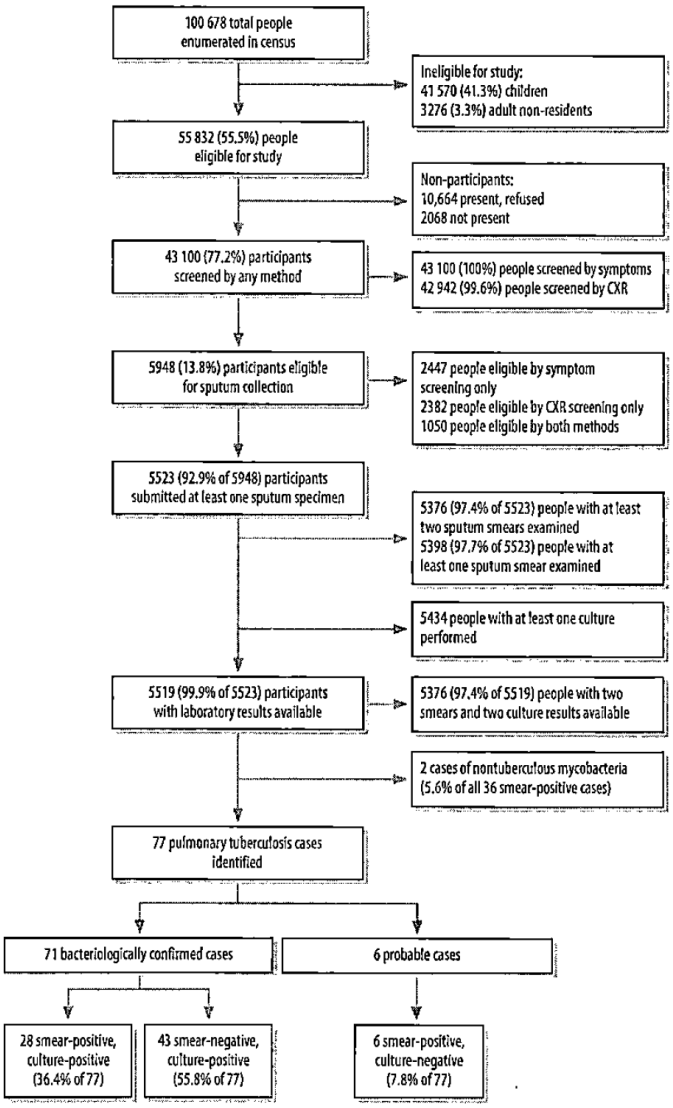
Most participants identified with tuberculosis (94.8%; 73) were not on treatment at the time of the survey and were not officially registered with the national tuberculosis programme. Thirty-eight patients had sought care on account of their symptoms; all 38 had visited health facilities and the great majority of them (31/38) had visited a public health facility.

Prevalence

There were missing data for smear, culture and symptom identification for 512 participants eligible for sputum examination; 24 of these had missing chest X-rays as well. Bacterial identification was a conditional imputation, conditioned on a positive culture. In addition, 134 subjects had only X-rays missing. In total, 646 subjects required imputation analysis.

For the adjusted prevalence estimates for smear-positive and bacteriologically confirmed pulmonary tuberculosis, the results from the three models were consistent and for models 2 and 3 were quite similar, especially for smear-positive tuberculosis (Table 1). Considering the larger differences for bacteriologically confirmed tuberculosis by residence and sex with model 3, the extrapolation by inverse probability weighting in this model appears to have adjusted for the lower

Fig. 3. **Outline of the national tuberculosis prevalence survey, Gambia, 2012**



CXR: chest X-ray.

Appendix

Table 1. **Adjusted overall estimated prevalence of pulmonary tuberculosis per 100 000 population aged ≥ 15 years, Gambia, 2012**

Group	Prevalence per 100 000 population (95% CI)					
	Smear-positive cases			Bacteriologically confirmed cases		
	Model 1[a]	Model 2[b]	Model 3[c]	Model 1[a]	Model 2[b]	Model 3[c]
Overall point estimate	80 (44–116)	92 (55–128)	90 (53–127)	181 (129–232)	199 (147–250)	212 (152–272)
Residence						
Rural	79 (27–132)	90 (44–142)	86 (32–140)	154 (90–219)	165 (102–228)	109 (54–164)
Urban	81 (35–127)	93 (44–142)	96 (43–148)	219 (138–301)	239 (152–327)	266 (164–368)
Sex						
Male	139 (82–195)	151 (88–213)	148 (88–208)	295 (208–381)	309 (221–396)	333 (233–433)
Female	40 (0–81)	40 (1–80)	41(0–83)	103 (50–155)	104 (53–156)	109 (54–164)
Age group (years)						
15–34	45 (19–71)	53 (23–82)	56 (24–88)	109 (63–155)	117 (70–163)	133 (76–190)
35–54	102 (57–190)	141 (59–224)	144 (65–223)	285 (178–392)	323 (199–447)	355 (219–490)
≥ 55	146 (55–387)	187 (0–385)	159 (0–367)	331 (92–570)	364 (140–588)	329 (99–558)

CI: confidence interval.
[a] Model 1: logistic regression model with robust standard errors and no missing value imputation.
[b] Model 2: logistic regression model with robust standard errors and missing value imputation of non-participants as well as participants.
[c] Model 3: logistic regression model with robust standard errors, with missing value imputation of participants with missing smear and/or culture results, and inverse probability weighting applied to all survey participants to correct for differentials in participation by age, sex and residence.[4,7]

Table 2. **Prevalence of pulmonary tuberculosis by sex and age group per 100 000 population aged ≥ 15 years, Gambia, 2012**

Group	Prevalence per 100 000 population (95% CI)[a]	
	Smear-positive cases	Bacteriologically confirmed cases
15–34 years		
Male	104 (42–166)	209 (113–305)
Female	33 (0–78)	71 (20–191)
35–54 years		
Male	282 (118–445)	634 (384–885)
Female	26 (0–80)	105 (19–191)
≥ 55 years		
Male	83 (0–203)	325 (108–543)
Female	227 (0–557)	330 (0–677)

CI: confidence interval.
[a] Model 3: robust standard errors with missing value imputation and inverse probability weighting; confidence intervals were calculated with exact binomial probability theory.

participation in urban areas and by males. This result suggests that model 3 has the best fit for our data. Based on model 3 the estimated prevalence of smear-positive tuberculosis was 90 per 100 000 population (95% CI: 53–127) and for bacteriologically confirmed tuberculosis was 212 per 100 000 (95% CI: 152–272).

Prevalent smear-positive tuberculosis was 3.4-fold higher in male than female participants. Bacteriologically confirmed pulmonary tuberculosis was 2.4-fold higher than smear-positive tuberculosis and 2.4-fold higher in urban than rural areas (Table 1). Table 2 shows age- and sex-associated differences in the burden of smear and bacteriologically confirmed tuberculosis based on estimates from model 3.

Adjusted prevalence

We calculated the adjusted tuberculosis prevalence for all ages and all forms of tuberculosis, with the assumptions that children aged < 15 years were 45.9% of the total population,[10] and that childhood tuberculosis and extrapulmonary tuberculosis were 10.5% and 6.7% of all total notifications, respectively. This produced a national prevalence for all age groups and all types of tuberculosis of 128 per 100 000 population (95% CI: 94–162). The updated incidence was 175 per 100 000 population (95% CI: 135–215) and the tuberculosis case notification rate was 130 per 100 000 population. Using routine country tuberculosis notification data, the smear positive tuberculosis prevalence, in the population aged < 15 years, was 3 per 100 000, the revised prevalence was 53 per 100 000.

Performance appraisal

Comparing the estimated case detection rates against global tuberculosis control targets showed that Gambia achieved the 70% case detection target over the period 2009–2013, except for the year 2010 (64.9%; Table 3).

Discussion

The overall prevalence of bacteriologically confirmed and smear-positive pulmonary tuberculosis in Gambia were 3.8- and 5.5-fold lower respectively than previously estimated in 2011.[2,3] These results are similar to those reported from Ethiopia, where the overall burden of tuberculosis was much lower than previously thought.[11] Results from other recently concluded surveys in Rwanda, Sudan and Zimbabwe, are also expected to report lower than expected prevalence.[1] However, recent surveys carried out in other parts of Africa (Ghana,[1] Malawi,[1] Nigeria,[12] the United Republic of Tanzania[1] and Zambia[12])

Appendix

Table 3. **Tuberculosis case detection rates in Gambia in the years 2009–2013 based on revised tuberculosis incidence estimates**

Group	Year				
	2009	2010	2011	2012	2013
Population[a]	1 681 734	1 728 394	1 776 103	1 824 777	1 882 450
Total no. of all types of notified tuberculosis[b]	2 065	1 962	2 249	2 333	2 340
Total no. of estimated incident cases[c]	2 943	3 024	3 108	3 193	3 294
No. of notified cases per 100 000 population	123	114	127	128	124
Case detection rate, % (95% CI)	70.2 (68.5–71.8)	64.9 (63.1–66.6)	72.4 (70.7–73.9)	73.1 (71.5–74.6)	71.0 (69.5–72.6)

CI: confidence interval
[a] Data source: Gambia 2003 National Census, Central Statistics Department, Government of the Gambia; other years are estimated values.
[b] Data source: routine reports from the Gambia National Tuberculosis and Leprosy Programme
[c] Estimated incidence is 175 per 100 000 population.

revealed higher than expected tuberculosis prevalence. Many of the earlier estimates of tuberculosis burden were derived from mathematical models. Therefore tuberculosis burdens could have been over- or underestimated, as actual measurements now suggest. For Gambia, it is unclear whether earlier figures were exaggerated or understated because of the varying pattern of survey results across Africa. This reinforces the need for reliable country-level measures of tuberculosis burden through surveys such as ours.

The majority of patients with tuberculosis identified in our study were unknown to the national tuberculosis control programme and were not on treatment. While passive case detection has well-known limitations, our data expose the importance of missed diagnostic opportunities within the routine health care system. This is not surprising given the weaknesses of health systems in low-and middle-income countries. While it remains unclear if active case-finding can interrupt tuberculosis transmission in the community through early diagnosis,[14] strengthening of case-finding through re-training staff and interventions such as the Practical Approach to Lung Health are essential.[15,16] Given that tuberculosis prevalence surveys are expensive undertakings (United States dollars 59 per participant in this survey), alternative approaches to active case-finding are required in resource-poor settings.

Tuberculosis was predominantly found in male participants in our study. The overall male-to-female ratio of 2.0:1 among participants identified with tuberculosis is within the range of ratios reported for Africa and all tuberculosis high-burden countries (0.5:1–3.0:1).[2] However, this conceals the fact that the male-to-female ratio of 3.7:1 for smear-positive tuberculosis in this survey exceeds the figures of 1.9:1 and 1.5:1 reported for the 22 countries with high-tuberculosis burden and the WHO African Region, respectively. In addition, the male-to-female ratio of 2.2:1 for all participants with bacteriologically confirmed tuberculosis exceeds the 1.7:1 and 1.3:1 reported for the tuberculosis high-burden countries and Africa. This sex difference was consistent across the survey case definitions, despite the lower participation by eligible males.

The majority of identified persons with tuberculosis were in the economically productive 15–44-year age group and this is an important finding for Gambia's economic well-being. Our survey also showed that tuberculosis prevalence increased with age, and, although the estimates were less precise for the oldest age group, our data points to the need for further investigation of tuberculosis among elderly people in Gambia. While the prevalence of smear-positive tuberculosis did not differ significantly by rural or urban area, urban participants were twice as likely to have bacteriologically confirmed tuberculosis. This suggests increased access to routine and improved tuberculosis diagnostic services especially TB cultures, and particularly in urban areas.

The finding that less than half of all people with tuberculosis would have been identified based on smear microscopy results alone highlights the limitation of this diagnostic approach. Furthermore, the additional number of participants with smear-positive samples identified on the basis of a positive chest X-ray highlights the limitation of a diagnostic algorithm based on a combination of symptoms and smear microscopy alone. Clearly, the use of X-ray in diagnostic algorithms would increase tuberculosis case notifications in Gambia. Our results also demonstrate the added benefit of using additional symptom categories to complement the most common tuberculosis symptom of chronic cough. More than half of participants with identified tuberculosis were smear-negative and culture-positive, indicating that additional diagnostic tools, e.g. rapid diagnostics tests and/or tuberculosis cultures, are needed for early diagnosis.

The clinical epidemiology of nontuberculous mycobacterial disease in most of sub-Saharan Africa and indeed many low and middle-income countries is not well described. In our survey, 5.6% of participants with smear-positive samples were confirmed by culture as due to nontuberculous mycobacteria, pointing to a risk of over-diagnosis of pulmonary tuberculosis under programmatic conditions and the consequent prescription of unnecessary tuberculosis therapy. This further highlights the limitations of smear microscopy as the single diagnostic modality for national tuberculosis programmes in low- and middle-income countries.

The tuberculosis case detection rate was around 70% for the 5 years preceding the survey. Considering the > 85% treatment success already achieved, the Gambia appears to have achieved the DOTS strategy targets for case detection and treatment. In addition, it has achieved the millennium development goal 6c for tuberculosis – to "have halted by 2015 and begun to reverse the incidence of malaria and other major diseases by 2015"[17] – and the Stop TB Partnership target for halving tuberculosis prevalence; as tuberculosis prevalence has decreased from 350 per 100 000 population in 1990 to 128 per 100 000 in 2013.[18,19] There have been substantial investments in tuberculosis control efforts in Gambia, including expansion of access to diagnosis and treatment, and various schemes to provide support to patients, all complemented by advocacy,

Appendix

communication and social mobilization efforts. However, the contribution made by the expanded DOTS programme to improved tuberculosis case detection and notification is unclear. While the DOTS strategy is credited for increasing tuberculosis detection and treatment success and in progress towards achieving global tuberculosis control targets,[20] other authors argue that it only improves treatment success.[21] The reduced burden of tuberculosis and the progress made via tuberculosis control efforts in Gambia, support the continued deployment of existing tuberculosis control strategies. However, the results of this survey highlight some gaps that need attention. For example, it is important to elucidate the factors associated with relatively stable tuberculosis notification rates despite achievement of the case detection and treatment success targets in the DOTS strategy.

This survey had some limitations. There were fewer participants than the target sample size but this was mainly the result of lower participation among males than females. Although robust statistical methods were applied to adjust for this, we might not have completely eliminated selection bias and/or the possibility that this survey underestimated the burden of tuberculosis in male participants. Despite the risk of reported sex differences being understated and the less precise estimates for some of the sub-group analyses, we believe the results here are valid because the observed sex differences are consistent with reports from other surveys and the literature. In addition, the survey was not designed to directly measure extrapulmonary or childhood tuberculosis. Although we did not offer voluntary testing and counselling for human immunodeficiency virus infection, such counselling is offered routinely to all newly diagnosed tuberculosis patients at tuberculosis treatment sites in Gambia, and uptake among newly diagnosed persons with tuberculosis exceeds 75%.[1,22]

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ملخص

مسح يتناول مدى انتشار مرض السل في غامبيا على مستوى الدولة بأكملها في عام 2012

الغرض تقدير مدى تفشي مرض السل الرئوي النشط بين السكان في غامبيا.

الطريقة خضع الأشخاص الذين تبلغ أعمارهم 15 عامًا أو أكثر ممن شاركوا في مسح عنقودي متعدد المراحل على مستوى الدولة بأكملها في الفترة بين ديسمبر/ كانون الأول 2011 ويناير 2013 لفحص للكشف عن الإصابة بالسل الرئوي النشط باستخدام تصوير الصدر بالأشعة وفحص للكشف عن أعراض السل. وللتأكد من التشخيص، تم جمع عينات من البصاق من الأشخاص الذين أظهر الفحص نتائج إيجابية لهم، وخضعت تلك العينات للفحص تحت المجهر الفلوري ولعمليات زراعة في وسط سائل للكشف عن بكتريا السل. وتمت الاستعانة بطريقة حساب القيم التعويضية المتعددة وأسلوب ترجيح الاحتمال العكسي في تقدير مدى تفشي مرض السل.

النتائج بلغ عدد الأشخاص المؤهلين للمشاركة 55832 شخص من بين الأشخاص الذين تم إحصاء أعدادهم والتي بلغت 100678، وقد شارك منهم بالفعل 43100 شخص (بنسبة 77.2٪). وخضع معظم المشاركين (42942؛ بنسبة 99.6٪) لإجراء ناجح للفحص الذي يكشف عن ظهور أعراض المرض وذلك الذي يعتمد على تصوير الصدر بالأشعة السينية. وبلغ عدد المؤهلين لاختبار البصاق 5948 شخصًا فقط (بنسبة 13.8٪)، حيث ظهر من بين هؤلاء الأشخاص حالات للإصابة بالسل تم تأكيد تشخيصها بالطرق البكتريولوجية لدى 43 شخصًا، وتأكدت النتائج الإيجابية لفحص المسحة لدى 28 شخصًا، وأظهر ستة أشخاص نتائج إيجابية محتملة لفحص المسحة تشير للإصابة بالمرض. وتم تحديد المزيد من حالات الإصابة بالسل (58/ 69) من خلال تصوير الصدر بالأشعة السينية مقارنة باستخدام الكشف عن ظهور الأعراض فقط (43/ 71). ووفقًا للتقديرات، فإن تفشي حالات الإصابة بالسل الرئوي التي تم تأكيد تشخيصها من خلال النتائج الإيجابية لفحص مسحة البصاق والطرق البكتريولوجية بلغ معدلًا مقداره 90 حالة (بنسبة أرجحية مقدارها 95٪: 53 – 127) و212 حالة (بنسبة أرجحية مقدارها 95٪: 152 – 272) في كل مجموعة سكانية يبلغ عدد أفرادها 100000 نسمة، على التوالي. وارتفعت نسبة تفشي مرض السل عند الذكور (333؛ بنسبة أرجحية مقدارها 95٪: 233 – 433) وكذلك في المجموعة العمرية من سن 35 إلى 54 عامًا (355؛ بنسبة أرجحية مقدارها 95٪: 219–490).

الاستنتاج يظل عبء مرض السل بمعدل مرتفع في غامبيا، إلا أنه يقل عن التقديرات السابقة التي أشارت إلى وجود 490 حالة في كل مجموعة سكانية يبلغ عدد أفرادها 100000 نسمة في عام 2010. وقد تم تحديد ما يقل عن النصف من الحالات بأكملها اعتمادًا على نتائج الفحص المجهري لمسحة البصاق وحده. وستتطلب الجهود الرامية للنجاح في السيطرة على المرض بعض التدخلات التي تستهدف الرجال، وزيادة فرص الخضوع للتصوير بالأشعة وتوفير المزيد من الاختبارات التشخيصية الدقيقة والسريعة.

Appendix

摘要

2012 年冈比亚全国范围内肺结核患病率调查

目的 旨在预测冈比亚境内活动性肺结核患病率。

方法 在 2011 年 12 月到 2013 年 1 月期间，研究人员采用 X 线胸片对年龄为 15 岁以及以上参与全国范围内、多阶段整群调查的人员进行活动性肺结核筛查以及肺结核症状筛查。为进行确诊，研究人员采集了筛查结果阳性人员的痰液样本并对其进行荧光显微镜观察以及肺结核液体培养基培养。还采用多重填补法和逆概率加权法以估算肺结核患病率。

结果 在选出的 100,678 人中，55,832 人有资格参与调查，其中 43,100 (77.2%) 人参与了调查。我们通过 X 线胸片成功地对大部分参与者（42,942 人；99.6%）进行了症状筛查。仅 5,948 (13.8%) 人有资格参加痰液检验，结果显示 43 人为细菌学检查阳性，28 人痰涂片检查为阳性，并且发现了六例痰涂片检查疑似阳性的肺结核病例。相对于单纯症状查痰法 (43/71)，X 线胸片可确诊更多肺结核病例 (58/69)。每 10 万人中，预计痰涂片阳性和细菌学检查阳性的肺结核患病人数分别为 90 (95% 置信区间，CI: 53-127) 和 212 (95% 置信区间，CI: 152 – 272)。男性 (333; 95% CI: 233 – 433) 以及年龄介于 35 – 54 岁的人群 (355; 95% CI: 219 – 490) 肺结核患病率更高。

结论 冈比亚境内肺结核患病率居高不下，但低于 2010 年估计的每 10 万人中 490 例的患病率。在所有病例中，半数以下病例本可以仅仅通过显微镜观察痰涂片确诊。为有效控制疾病发展，需要对男性采取干预措施、进一步促进 X 线胸片以及更多精确、快速诊断检测方法的普及。

Résumé

Enquête sur la prévalence de la tuberculose à l'échelle nationale en Gambie en 2012

Objectif Estimer la prévalence de la tuberculose pulmonaire active dans la population de Gambie.

Méthodes Entre décembre 2011 et janvier 2013, dans le cadre d'une enquête nationale en grappes à plusieurs degrés, un dépistage de la tuberculose pulmonaire active a été réalisé au moyen d'un questionnaire sur les symptômes tuberculeux et d'une radiographie pulmonaire, chez des personnes âgées de 15 ans ou plus ayant consenti à participer. Pour confirmer le diagnostic, des échantillons d'expectoration ont été collectés pour tous les participants dont les résultats de dépistage s'étaient révélés positifs, en vue de réaliser des examens par microscopie de fluorescence et des cultures en milieu liquide. Les techniques d'imputation multiple et de pondération par l'inverse de la probabilité ont été utilisées pour estimer la prévalence de la tuberculose.

Résultats Sur les 100 678 personnes recensées, 55 832 étaient éligibles pour participer à l'étude et, parmi elles, 43 100 personnes (77,2%) ont participé. La majorité des participants (42 942; 99,6%) ont effectivement répondu au questionnaire sur les symptômes et passé une radiographie pulmonaire. Seules 5 948 personnes (13,8%) ont été éligibles pour un examen des expectorations, ce qui a entraîné le dépistage de 43 cas de tuberculose confirmée bactériologiquement, de 28 cas formels de tuberculose à frottis positif et de 6 cas probables de tuberculose à frottis positif. Les radiographies pulmonaires ont permis d'identifier plus de cas de tuberculose (58/69) que le seul questionnaire sur les symptômes (43/71). Les prévalences de la tuberculose pulmonaire à frottis positif et de la tuberculose bactériologiquement confirmée ont respectivement été estimées à 90 cas (intervalle de confiance de 95%: 53–127) pour 100 000 habitants et 212 cas (IC 95%: 152–272) pour 100 000 habitants. La prévalence de la tuberculose s'est avérée plus élevée dans la population masculine (333 cas; IC 95%: 233–433) et dans la tranche des 35–54 ans (355; IC 95%: 219-490).

Conclusion La charge de la tuberculose reste élevée en Gambie, même si elle a baissé par rapport à la précédente estimation, réalisée en 2010, qui faisait état de 490 cas pour 100 000 habitants. En utilisant uniquement la microscopie des frottis, moins de la moitié des cas auraient été identifiés. Pour être efficaces, les efforts de lutte contre la tuberculose doivent inclure des campagnes ciblant les hommes, améliorer l'accessibilité des examens radiographiques et utiliser des tests diagnostiques rapides plus précis.

Резюме

Исследование распространенности туберкулеза в национальном масштабе — Гамбия, 2012 год

Цель Оценить популяционную распространенность активной формы туберкулеза легких в Гамбии.

Методы В период с декабря 2011 года по январь 2013 года лица в возрасте ≥ 15 лет принимали участие в национальном многоэтапном исследовании с применением гнездовой выборки; их обследовали на наличие активной формы туберкулеза легких при помощи рентгенографии грудной клетки, а также проверяли наличие у них клинических симптомов туберкулеза. С целью подтверждения диагноза у лиц, для которых результаты скринингового обследования были положительными, брали пробу мокроты и подвергали ее флуоресцентной микроскопии, а также применяли ее посев на жидкую питательную среду. Для оценки распространенности туберкулеза применялся метод множественного восстановления пропущенных данных и метод весовых коэффициентов для величин, обратных вероятности.

Результаты Из 100 678 человек, зарегистрированных для проведения обследования, 55 832 отвечали критериям исследования. Из них 43 100 человек (77,2%) приняли участие в исследовании. В большинстве своем участники (42 942 (99,6%)) успешно прошли скрининг симптомов и рентгенографию грудной клетки. Только 5 948 участников (13,8%) отвечали критериям для сдачи мокроты на анализ, из них в 43 случаях было получено бактериологическое подтверждение, 28 мазков были квалифицированы как достоверно положительные, шесть — как вероятные случаи туберкулеза. Рентгенография грудной клетки выявила больше случаев туберкулеза (58 из 69), чем диагностика только по симптомам (43 из 71). Оценка распространенности положительного мазка на туберкулез и бактериологически подтвержденного туберкулеза легких составила 90 (95%-й доверительный интервал, ДИ: 53–127) и 212 (95%-й ДИ: 152–272) на 100 000 населения соответственно. Туберкулез чаще

Appendix

встречался среди мужчин (333, 95%-й ДИ: 233–433) и лиц в возрасте 35–54 лет (355, 95%-й ДИ: 219–490).
Вывод В Гамбии сохраняется высокое бремя туберкулеза, но полученные оценочные значения ниже величины, рассчитанной в 2010 году, в 490 случаях на 100 000 населения. Менее половины всех случаев могли быть выявлены только на основании микроскопического исследования мазка. Для успеха контролирующих мероприятий потребуются вмешательства, ориентированные на мужчин, более широкий доступ к рентгенографии и более точные и быстродействующие диагностические тесты.

Resumen

Una encuesta nacional de prevalencia de la tuberculosis en Gambia, 2012

Objetivo Estimar la prevalencia de la tuberculosis pulmonar activa entre la población en Gambia.
Métodos Entre diciembre de 2011 y enero de 2013, personas de 15 años o más que participaron en una encuesta nacional en varias etapas y a nivel de grupos fueron examinadas en busca de tuberculosis pulmonar activa mediante una radiografía de tórax y para encontrar síntomas de tuberculosis. Para confirmar el diagnóstico, se recolectaron muestras de esputo de las personas cuyo examen resultó positivo y se sometieron a microscopia de fluorescencia y cultivos líquidos de tuberculosis. Para calcular la prevalencia de la tuberculosis, se utilizaron varias asignaciones y análisis de probabilidad inversa.
Resultados De las 100 678 personas enumeradas, 55 832 se consideraron aptas para participar, de las cuales 43 100 (77,2%) participaron. La mayoría de los participantes (42 942; 99,6%) fueron examinados con éxito para detectar síntomas, así como por radiografía del tórax. Solo 5 948 (13,8%) fueron aptos para pruebas de esputo, lo que dio lugar a 43 confirmados a nivel bacteriológico, 28 casos bacilíferos de tuberculosis definitivos y 6 casos bacilíferos de tuberculosis probable. Las radiografías del tórax identificaron más casos de tuberculosis (58/69) que los síntomas por sí solos (43/71). La prevalencia estimada de tuberculosis pulmonar bacilífera y confirmada a nivel bacteriológico fue de 90 (intervalo de confianza, IC, del 95%: 53–127) y 212 (IC del 95%: 152–272) por cada 100 000 habitantes, respectivamente. La prevalencia de la tuberculosis fue mayor en hombres (333; IC del 95%: 233–433) y en los grupos de edad entre 35 y 54 años (355; IC del 95%: 219–490).
Conclusión Los índices de tuberculosis siguen siendo altos en Gambia, pero inferiores a las estimaciones anteriores de 490 por cada 100 000 habitantes en 2010. Se identificaron menos de la mitad de todos los casos según los resultados de la microscopia de frotis por sí solos. Para que los esfuerzos de control tengan éxito, serán necesarias intervenciones enfocadas a los hombres, un mayor acceso a radiografías y pruebas de diagnóstico más rápidas y exactas.

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PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Impact of the *Mycobaterium africanum* West Africa 2 Lineage on TB Diagnostics in West Africa: Decreased Sensitivity of Rapid Identification Tests in The Gambia

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Abstract

Background

MPT64 rapid speciation tests are increasingly being used in diagnosis of tuberculosis (TB). *Mycobacterium africanum* West Africa 2 (*Maf 2*) remains an important cause of TB in West Africa and causes one third of disease in The Gambia. Since the introduction of MPT64 antigen tests, a higher than expected rate of suspected non-tuberculous mycobacteria (NTM) was seen among AFB smear positive TB suspects, which led us to prospectively assess sensitivity of the MPT64 antigen test in our setting.

Methodology/Principal Findings

We compared the abundance of mRNA encoded by the *mpt64* gene in sputa of patients with untreated pulmonary TB caused by *Maf* 2 and *Mycobacterium tuberculosis (Mtb)*. Subsequently, prospectively collected sputum samples from presumptive TB patients were inoculated in the BACTEC MGIT 960 System. One hundred and seventy-three acid fast bacilli (AFB)-positive and blood agar negative MGIT cultures were included in the study. Cultures were tested on the day of MGIT positivity with the BD MGIT TBc Identification Test. A random set of positives and all negatives were additionally tested with the SD Bioline Ag MPT64 Rapid. MPT64 negative cultures were further incubated at 37°C and retested until positive. Bacteria were spoligotyped and assigned to different lineages. *Maf* 2 isolates were 2.52-fold less likely to produce a positive test result and sensitivity ranged from 78.4% to

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84.3% at the beginning and end of the recommended 10 day testing window, respectively. There was no significant difference between the tests. We further showed that the decreased rapid test sensitivity was attributable to variations in mycobacterial growth behavior and the smear grades of the patient.

Conclusions/Significance

In areas where *Maf 2* is endemic MPT64 tests should be cautiously used and MPT64 negative results confirmed by a second technique, such as nucleic acid amplification tests, to avoid their misclassification as NTMs.

Author Summary

Diagnostics for rapid confirmation of positive liquid cultures presumptive of *Mycobacterium tuberculosis* bacteria, based on the detection of the MPT64 antigen, are being used in many TB diagnostic laboratories worldwide. Of note, diagnostic performance of these tests in West Africa, where TB is uniquely caused by the geographically restricted *Mycobacterium africanum* (*Maf 1* and *2*) and *Mycobacterium tuberculosis* lineages, has not been properly assessed. Although *M. tuberculosis* and *M. africanum* are genetically related, they differ in various aspects. Amongst several differences, *Maf 2* grows significantly slower than *Mtb* bacteria. Because secretion of the MTP64 protein is dependent on the bacterial growth rate, we found that the MPT64 rapid test performance for detecting *Maf 2* was lower in our setting in The Gambia. These findings might be relevant for other West African *Maf 2* endemic countries where this rapid test is commonly used, as *Maf 2* infected patients might have been missed in the past. Our finding emphasizes the need to thoroughly consider the presence of bacterial variants specific to certain regions during product development and implementation of novel diagnostic tests.

Introduction

Tuberculosis remains a significant public health problem in Africa. Key to interrupting transmission, which is an essential step to reduce the incidence of TB, is timely identification and treatment of diseased individuals. This objective has fueled research into new generations of diagnostics and drugs. MPT64 is a 24kD secreted protein that has been explored in diagnostics and vaccine design due to several properties: it has been associated with virulence, is highly immunogenic and is produced solely by members of the *Mycobacterium tuberculosis* complex [1,2,3]. MPT64 is the target of three widely used rapid speciation lateral flow assays for the identification of the MTBc in culture; BD MGIT TBc Identification Test (BD TBc ID) (Becton Dickinson Diagnostics, Becton, Dickinson and Company, Sparks, Maryland, USA), SD Bioline Ag MPT64 Rapid (SD Bioline) (Standard diagnostics, Inc., Yongin-si, Gyeonggi-do, Republic of Korea), and Capilia TB-Neo (TAUNS Laboratories, Inc., Numazu, Shizuoka, Japan). Despite the advantage of a lateral flow assay, there have been reports of the failure of MPT64 tests to detect MTBc isolates, resulting in erroneous reporting of Non-tuberculous mycobacteria (NTM) isolation [2,3,4,5]. Inability to identify the MTBc will lead to delays in initiating appropriate treatment with dire consequences for the patient and their communities given the continued risk of TB transmission.

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The global phylogeographical distribution of the MTBc suggests that strain diversity is greatest in West Africa, with a representation of all major MTBc lineages [6]. Differences between these MTBc lineages might affect the performance of diagnostics and vaccines [7]. Thus, West Africa is ideal for providing a global snapshot of the performance of TB diagnostics and vaccines. Interestingly, few studies assessing commercially available MPT64 MTBc rapid speciation tests were undertaken within African populations. They were mostly done in Asia, America and Europe where strain diversity is significantly lower [8].

Lineage 6, *Mycobacterium africanum* (*Maf*) West African 2, is geographically restricted to West Africa, causing up to one third of clinically reported TB. We and others have previously described inherent genotypic and phenotypic differences between *Mycobacterium tuberculosis* sensu stricto (*Mtb*) and *Maf 2* strains *in vitro* and within the host [9,10,11,12]. Since we previously observed differences in various virulence factors between *Maf 2* and *Mtb* and MPT64 is a described virulence factor, we hypothesized that the sensitivity of MPT64 rapid tests for MTBc identification could be different for *Maf 2* relative to *Mtb* strains.

In The Gambia, where *Maf* 2 is commonly isolated, we compared the abundance of mRNA encoded by the *mpt64* gene in *Maf* 2 strains versus *Mtb* strains in the sputa of untreated TB patients. The *mpt64* (Rv1980c) mRNA transcript was significantly less abundant in the sputa of TB patients infected with *Maf* 2 compared with *Mtb*. Therefore we concluded that *Maf 2* might either produce the MPT64 protein at a slower rate and possibly below the limit of detection of the rapid tests. We confirmed this hypothesis and found a reduced *Maf 2* sensitivity of rapid tests. Further, we compared the time to detection of the MPT64 antigen by the BD TBc ID and SD Bioline rapid tests between clinical isolates of *Mtb* and *Maf 2*. We report lineage dependent and time specific differences in conversion to MPT64 test positivity. Our findings have direct implications on the performance of these and other MPT64 based tools in West Africa.

Materials and Methods

Ethics Statement

This study was nested within an intervention trial of Enhanced Case Finding in The Gambia (Clinicaltrials.gov NCT01660646). The parent study, including bacterial sub-studies, received ethical approval from the Joint Gambia Government/MRC Ethics Committee and the Institute of Tropical Medicine (ITM), Antwerp Institutional Review Board. Written informed consent was obtained from all participants who were assigned unique identifiers for purposes of anonymity and confidentiality.

Microscopy and Decontamination of Sputum

Sputum samples were prospectively collected from individuals with suspected TB between April and October 2014 and were all initially screened for the presence of AFB by Auramine microscopy. Fresh samples were decontaminated by the NALC-NaOH method as described previously [13]. The purity of all decontaminated samples was subsequently checked on blood agar for 48 hours at 37°C and screened for AFB by Ziehl-Neelsen [14] staining, during which time the decontaminated sputa were stored at -20°C, prior to inoculation.

Mtb Gene Expression Analysis in Sputum

Sputa from 11 adult patients with smear positive TB that had not started therapy were collected and stored in Guanidine Isothiocyanate. Later 5 *Maf 2* and 6 *Mtb* sputum specimens were re-suspended in Trizol for total RNA isolation. Gene expression of *mpt64* gene was performed

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using Multiplex qPCR with previously published TaqMan primer-probe sets as described previously [15,16]. Multiplex RT-PCR data were normalized and analysed according to a previously published method. For a detailed description please refer to Garcia *et al.* [17].

Bacterial Culture

Confirmed blood agar negative and AFB positive samples were cultured within the BACTEC MGIT 960 System (MGIT 960; Becton Dickinson Microbiology Systems, Sparks, Maryland, USA) at 37°C according to the manufactures' instructions. Instrument positive vials were removed from the machine and again subjected to purity check using blood agar and ZN microscopy to confirm the presence of AFB. Cultures with growth on blood agar and/or AFB negative were excluded from the study.

MPT64 Antigen Test

Blood agar negative and AFB positive MGIT cultures were tested on the day of MGIT positivity (day 0, T_0) with the BD TBc ID rapid speciation lateral flow assay, following the manufacturers' instructions. The BD TBc ID manual specifies that tests should be performed on AFB-positive MGIT tubes only if AFB-positive organisms predominate on a smear and cautions on the possibility of false results due to the presence of non-AFB organisms in cultures. In order to test whether the lower sensitivity of the MPT64 assay was specific to one manufacturer, we additionally tested all BD TBc ID negative samples at T_0, including a random number of positive samples, with the SD Bioline kit at the same time points. Tests were always independently evaluated by at least two blinded readers; in case of discordance between the readers a third blinded reader was consulted. Faint bands were considered as positive results. Results were recorded after 15 minutes and negative cultures at T_0 were retested at 3, 10, 15 and 90 days with both the BD TBc ID and SD Bioline rapid speciation kits.

Spoligotyping

Aliquots of the MGIT cultures were taken at T_0 and heat killed. These lysates were genotyped by spoligotyping as previously described [18]. Patient isolates were assigned to specific TB lineages using the TB-lineage tool [19] within the TB-Insight public database.

Statistical Analysis

We plotted the survival curves for *Maf* 2 and *Mtb* conversion to rapid test positivity at 1, 3, 10, 15 and 90 days and used the generalized Log-rank test for interval-censored failure time [20] to compare the two survivor functions. We estimated the effect of MTBc lineage (*Maf 2* and *Mtb*) on the time-to-conversion to rapid test positivity using a Weibull regression model for interval-censored failure time [20] after controlling for testing time interval, age, gender, smear grade, duration spent in the MGIT, mycobacterial growth units, and whether patients had already initiated therapy. The BD TBc ID manual indicated that tests could be performed within 10 days after MGIT tube positivity, while SD Bioline did not specify any time interval for the performance of tests. Therefore, for comparative evaluation we stratified both analyses by a 0–10 days testing window and 10–90 days follow-up. A paired interval-censored analysis was used to compare BD TBc ID and SD Bioline performance. All analyses were performed using STATA 13.1 (Stata Corp., College Station, Texas).

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Results

Lower Expression of the *mpt64* Transcript in the Sputa of *M. africanum* Compared to *M. tuberculosis* TB Patients

On comparing the abundance of the *mtp64* mRNA transcript in sputum samples from 6 *Mtb* and 5 *Maf 2* infected patients, who had not received any TB treatment, the *mpt64* transcript was significantly less abundant in the sputa of *Maf 2*-infected patients, compared to sputa of *Mtb*-infected patients (fold change = 2.52, p = 0.006). To validate our expression data, we measured the abundance of mRNA transcripts of the gene (Rv2355) responsible for the transposition of IS6110 elements in *Maf 2* samples since *Maf 2* is known to have lower copy numbers of IS6110 elements than *Mtb* [21]. Interestingly, we found a significantly reduced abundance of mRNA transcripts of this transposase in *Maf 2* samples. *In vitro* under expression of the polyketide synthesis loci responsible for production of sulfolipids in *Maf 2* has been reported [22], which were also significantly downregulated in *Maf 2* in our *ex vivo* data. For a detailed overview of mRNA expression for each individual patient please see S3 Table.

Culture of Bacteria

Altogether, 193 sputum samples from 193 presumptive TB patients were cultured in the MGIT 960 System. For a summary of patients' characteristics see Table 1. Of the 193 cultures, 20 (10.4%) were excluded from the study due to missing patient data (n = 4; (2.1%)), being AFB negative and contaminated with other microorganisms on blood agar (n = 4; (2.1%)), AFB negative without contamination (n = 9; (4.7%)), or AFB positive and contaminated (n = 3; (1.5%)) (see Fig 1).

A hundred and seventy-three positive cultures (89.6%), from 168 AFB smear positive and 5 AFB smear negative sputa, were blood agar negative, AFB positive by ZN staining and were tested with the BD TBc ID kit (Fig 1). One hundred and fifty samples tested positive with the BD TBc ID speciation kit on T_0, with 23 testing negative at this time point, representing 13.2% of cultures tested.

Table 1. Clinical and demographic characteristics of study participants.

	Maf 2	*Mtb*
	(n = 51)	(n = 122)
Age, median (range)	30 (17–67)	30 (15–90)
Gender		
Male, n (%)	46 (90.2)	79 (64.8)
Female, n (%)	5 (9.8)	43 (35.2)
Smear grade, n (%)		
Scanty	4 (7.8)	15 (12.3)
+	14 (27.5)	28 (23.0)
++	11 (21.6)	37 (30.3)
+++	22 (43.1)	42 (34.4)
Therapy, n (%)		
Yes	24 (47.1)	60 (49.2)
No	10 (19.6)	18 (14.8)
Unknown	17 (33.3)	44 (36.1)

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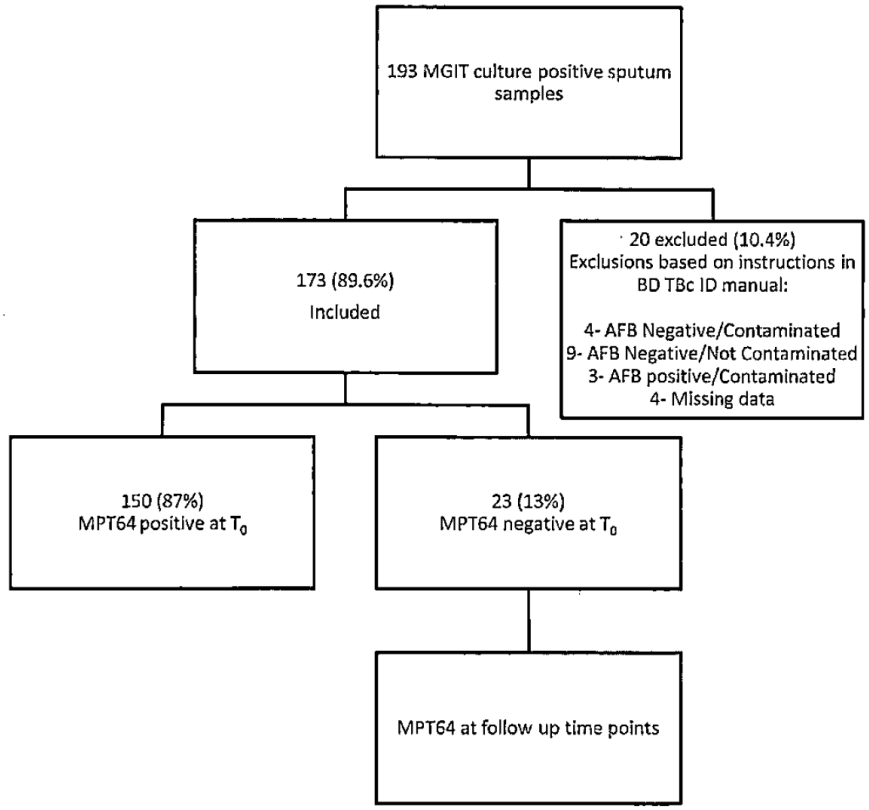

Fig 1. Flowchart describing sample collection and processing.

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Lower Sensitivity of MPT64 Tests for *M. africanum* than *M. tuberculosis*

All 173 samples were spoligotyped and assigned to a lineage within the MTBc. Samples genotyped belonged to lineages 1 (Indo-oceanic), 2 (East-Asian/Beijing), 4 (Euro-American) or 6 (*Maf 2*).

The conversion time of the BD TBc ID assay did not differ among the three *Mtb* lineages-East-Asian, Euro-American and Indo-Oceanic (East-Asian and Euro-American (p = 0.55), East-Asian and Indo-Oceanic (p = 0.89) and Euro-American and Indo-Oceanic (p = 0.63) (S1 Table). Therefore, we combined the three lineages into one group, *Mtb*.

We found an overall reduced rapid test sensitivity for *Maf 2* when compared to *Mtb* (see Fig 2 and Table 2).

There was strong evidence of a difference in the time to detection between *Maf 2* and *Mtb* (P = 0.001), with *Mtb* strains having a higher rate of conversion to BD TBc ID rapid test positivity than *Maf 2* (Fig 2 and Table 2).

After controlling for age, gender, smear grade, duration spent in the MGIT automated culture system, mycobacterial growth units, and whether patients had already received some TB therapy, in a multivariable analysis, there was strong evidence of association between species

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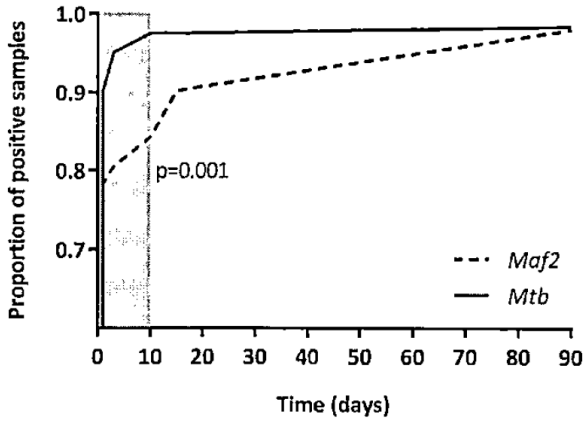

Fig 2. Kaplan-Meier survival plot showing the difference in conversion to rapid test positivity for *Mtb* strains and *Maf 2*. The Log-rank test was used to compare the survivor functions of *Mtb* and *Maf 2*. The gray shaded area indicates the sampling time window recommended by the manufacturer.

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and conversion to BD TBc ID positivity during the first 10 days ($p<0.0001$, Table 3). The rate of rapid test positivity in *Mtb*-infected patients was almost 4 times higher compared to *Maf 2*-infected patients within the first 10 days. From 10–90 days, there was no evidence of difference in the time to a positive test between *Mtb* and *Maf 2* ($p = 0.15$, Table 3).

There was strong evidence of association between smear grade and conversion to positivity ($p = 0.0001$, Table 3) after controlling for lineage, testing time interval, gender, age, therapy, duration spent in the MGIT and mycobacterial growth units. The rate of conversion to positivity in patients having smear grade +++ was about 3 times higher than patients with a scanty smear. Generally, cultures that spent longer in the MGIT automated culture system before turning culture positive were more likely to have a BD TBc ID positive rapid test result at T_0 ($p<0.0001$).

As shown in Table 3, there were no associations between conversion to BD TBc ID positivity and gender, age, therapy or mycobacterial growth units.

As only a subset of MGIT cultures were tested with both MPT64 assays, firstly, we assessed if there was any evidence of selection bias. Finding none (S2 Table), we compared their performance

Table 2. Percentage of samples converting to positivity over time using BD TBc ID.

	% of positive samples (95% CI)		p#
	Maf 2	*Mtb*	
	(n = 51)	(n = 122)	
BD TBc ID			
Day 0 (T_0)	78.4 (65.4–87.5)	90.2 (83.6–94.3)	0.04
Day 3 (T_3)	80.4 (67.5–89.0)	95.1 (89.7–97.7)	0.002
Day 10 (T_{10})	84.3 (72.0–91.8)	97.5 (93.0–99.2)	0.001
Day 15 (T_{15})	90.2 (79.0–95.7)	97.5 (93.0–99.2)	0.04
Day 90 (T_{90})	98.0 (89.7–99.7)	98.4 (94.2–99.5)	0.88

Two-sample test for proportions.

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Table 3. Multivariable analysis of BD TBc ID conversion time to positivity using interval-censored failure time regression using Weibull distribution. Each individual regression was adjusted for all other variables.

		Adjusted HR t (95% CI)	p
Testing time interval			
0–10 days	*Maf 2*	1	-
	Mtb	3.47 (1.75–6.88)	<0.0001
10–90 days	*Maf 2*	1	-
	Mtb	0.33(0.08–1.48)	0.15
Gender	Female	1	-
	Male	0.83 (0.49–1.40)	0.49
Age		0.99 (0.97–1.01)	0.13
Smear grade			0.01[a]
	Scanty	1	-
	+	1.31 (0.64–2.68)	0.47
	++	1.29 (0.63–2.63)	0.49
	+++	2.91 (1.38–6.14)	0.005
Therapy	No	1	-
	Yes	0.95 (0.62–1.46)	0.82
Time to MGIT positivity (h)		1.004 (1.002–1.004)	<0.0001
MGIT Growth units		1.00 (0.99–1.01)	0.33

[a] Wald test assessing evidence of overall association between smear grade and time-to-conversion.

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in a paired interval-censored analysis restricted to samples with results from both assays, correcting for the same explanatory variables as used in the BD TBc ID survival analysis.

Within each time interval and for each strain, there was no evidence of difference in time to rapid test positivity between BD TBc ID and SD Bioline (Table 4), suggesting that the decreased sensitivity may not be manufacturer specific yet intrinsic to the MPT64 target.

Discussion

Our findings suggest 2.5-fold decreased expression of the *mpt64* gene in Lineage 6 of *M. africanum* compared to *M. tuberculosis* and a significant decrease in sensitivity (78% on day T_0) of the MPT64 based lateral flow assays for speciation of Lineage 6 cultures relative to *M. tuberculosis*, as members of the MTBc.

Among the smear microscopy positive TB suspects enrolled in the present study, all were ultimately confirmed as MTBc infected, although 22% of *Maf* 2 patients, and 10% of MTB patients, would have been misclassified as NTMs if the tests had not been repeated after T_0, the day the MGIT culture turned positive. During the 10-day MGIT positive window recommended by the BD TBc ID manufacturer, only 84% of all *Maf 2* were detected by a positive test versus 98% of *Mtb* strains. Given the relatively low cost, limited technical expertise and shorter turnaround time associated with using rapid speciation tests compared to alternative speciation methods, MPT64 rapid tests will likely remain one of the preferred options for timely diagnosis of suspected TB despite the possibility of false negative results. Therefore, a negative MPT64 result would require confirmation by an alternative method, such as molecular tests or culture on para-nitrobenzoic acid (PNB), depending on laboratory infrastructure and resources. As BD and SD Bioline MPT64 rapid tests have been on the market for over a decade now, several groups have now evaluated them. The inability of the MPT64 tests to detect strains that have

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Table 4. Paired interval-censored survival analysis of conversion to positivity between BD TBc ID and SD.

	HR adjusted for testing time point (95% CI)*	p
Conversion to rapid test positivity in *Maf*		
0–10 days		
TBc	1	
SD	0.60 (0.35–1.01)	0.06
10–90 days		
TBc	1	
SD	0.86 (0.47–1.56)	0.62
Conversion to rapid test positivity in *Mtb*		
0–10 days		
TBc	1	
SD	1.39 (0.72–2.68)	0.33
10–90 days		
TBc	1	
SD	1.39 (0.72–2.68)	0.33

*: All estimates were adjusted for gender, age, therapy, smear grade, time to MGIT positivity and MGIT Growth units.

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lost the *mpt64* gene or acquired mutations has been reported previously [4,23]. A recent meta-analysis reported a sensitivity ≥ 95% yet studies evaluating these tests were conducted using a very limited panel of MTBc lineages, the majority belonging to *M. tuberculosis sensu stricto* lineages.

Two studies including *Maf* were biased by the low number of isolates evaluated compared to *Mtb* strains tested [24,25]. In their analysis of the sensitivity of the BD TBc ID test using reference strains, Yu *et al* included one *Maf* strain among 24 NTM strains, 18 mixtures of *M. tuberculosis* and NTM strains, 2 *M. bovis* strains and 1 *Nocardia spp.* strain. In the same study, 171 clinical respiratory specimen were prospectively analyzed yet these were all *M. tuberculosis* isolates [25]. Gaillard and co-workers, in their assessment of both the SD Bioline and BD TBc ID tests, included 20 *Maf* among 318 MTBc consisting of 242 *M. tuberculosis*, 53 *M. canettii*, 2 *M. bovis* and 1 *M. bovis* BCG Pasteur [24]. Although all *Maf* strains were detected, neither of the studies specified whether *Maf 1* or *Maf 2* isolates were tested. Interestingly, an earlier report hinted on the possibility of MPT64 rapid test sensitivity being influenced by the amount of antigen secreted by the metabolically growing MTBc cells and emphasised the need to determine factors driving secretion [26]. Notably, Gaillard and co-workers reported the production of a faint band after the standard 15 min incubation period by 8 out of 20 *Maf* strains tested during their evaluation of both the SD Bioline and BD TBc ID tests. They suggested further incubation of cultures which produce faint bands to allow production of greater amounts of antigen by the MTBc strain that could be clearly detected as positive [24]. Interestingly, in this study, we also observed an association between the duration spent by cultures in the MGIT automated culture system and positive rapid test results. The longer cultures spent in the MGIT culture system, the more likely they were to produce detectable amounts of MPT64 and therefore a positive rapid test result. Furthermore, Gagneux and colleagues detected a non-synonymous SNP in the *mpt64* gene of all isolates from *Maf 1*, which they hypothesized could affect the sensitivity of MPT64 tests in West Africa [7]. As no *Maf 1* was identified in our study, the sensitivity of the MPT64 rapid tests to detect *Maf 1* will need to be validated

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elsewhere. However, in a report from Nigeria, no failures of the MPT64 test were reported in detecting *Maf 1*, albeit not all positive cultures were accounted for [27].

One possible explanation for the evidence of association between high smear grade and rapid test positivity could be the presence of a greater number of actively dividing cells secreting the MPT64 antigen leading to an abundance of antigen in the growth medium. Interestingly, *Mtb* strains reportedly divide significantly faster than *Maf 2* strains [9]. Since the secretion of MPT64 has previously been linked to active cell division [1] the differential growth behaviour could also support our finding of lower detection of MPT64 rapid tests for *Maf 2*. Additionally future studies should ascertain if the observed under expression of *mpt64* in *Maf 2* was due to mutations in the gene. Whole genome sequencing will also enable us to confirm if these *Maf* 2 strains belong to a unique clone that is currently spreading in the Gambia or whether it is a general trait of *Maf 2*.

Conclusions

Our findings indicate that MPT64 tests need to be cautiously used in settings where *Maf 2* is common. Different modifications of workflow can be considered, such as repeating the MPT64 assay after 10 days on all T_0 MPT64 negative cultures, and molecular confirmation as MTBc vs NTMs in AFB positive cultures that test MPT64 negative. A preferred approach will be to apply the Xpert MTB/RIF assay or Line-Probe-Assays (LPA) to all AFB positive, MPT64 negative cultures. Generally, our findings strongly emphasize the need to consider strain diversity during TB product development. Our study further demonstrates that a careful evaluation and validation of novel tests before implementation, especially in regions with geographically restricted MTBC lineages, such as *M. africanum* in West Africa, is imperative.

Supporting Information

S1 Table. Comparison of survivor functions of the three *Mtb* lineages; East-Asian (Lineage 2), Euro-American (Lineage 4) and Indo-Oceanic (Lineage 1) for the BD TBc ID.
(DOCX)

S2 Table. Comparison of clinical and demographic characteristics of study participants whose samples were positive with BD TBc ID at T_0 and were also re-tested with SD.
(DOCX)

S3 Table. mRNA expression and ct values for the polyketide synthesis loci and the IS6110 transposase.
(DOCX)

Author Contributions

Conceived and designed the experiments: FG BCdJ BOA GS GD. Performed the experiments: BOA AS FK CO. Analyzed the data: AS BOA FG SCA. Contributed reagents/materials/analysis tools: OS MA IA. Wrote the paper: FG BOA BCdJ.

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Non-tuberculous Mycobacteria isolated from Pulmonary samples in sub-Saharan Africa - A Systematic Review and Meta Analyses

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Abstract

Pulmonary non-tuberculous mycobacterial (NTM) disease epidemiology in sub-Saharan Africa is not as well described as for pulmonary tuberculosis. Earlier reviews of global NTM epidemiology only included subject-level data from one sub-Saharan Africa country. We systematically reviewed the literature and searched PubMed, Embase, Popline, OVID and Africa Wide Information for articles on prevalence and clinical relevance of NTM detection in pulmonary samples in sub-Saharan Africa. We applied the American Thoracic Society/Infectious Disease Society of America criteria to differentiate between colonisation and disease. Only 37 articles from 373 citations met our inclusion criteria. The prevalence of pulmonary NTM colonization was 7.5% (95% CI: 7.2% - 7.8%), and 75.0% (2325 of 3096) occurred in males, 16.5% (512 of 3096) in those previously treated for tuberculosis and *Mycobacterium avium* complex predominated (27.7% [95% CI: 27.2 – 28.9%]). In seven eligible studies, 27.9% (266 of 952) of participants had pulmonary NTM disease and *M. kansasii* with a prevalence of 69.2% [95% CI: 63.2 – 74.7%] was the most common cause of pulmonary NTM disease. NTM species were unidentifiable in 29.2% [2,623 of 8,980] of isolates. In conclusion, pulmonary NTM disease is a neglected and emerging public health disease and enhanced surveillance is required.

Introduction

The epidemiology of pulmonary disease caused by *Mycobacterium tuberculosis* complex (MTBC) - *M. tuberculosis, M. bovis, M. africanum, M. canetti, M. microti, M. pinnipedii* and *M. caprae* - is better known than for NTM [1]. NTM is a designation used for a large number of potentially pathogenic and non-pathogenic environmental mycobacterial species other than MTBC and *Mycobacterium leprae*.

Worldwide, pulmonary infections caused by NTM are gaining increased attention, in part, because of their increasing recognition and isolation in clinical settings, for example with better known NTM pathogens such as *M. avium subsp paratuberculosis* , *M. marinum*, etc. [2,3]. Although NTM were identified soon after Koch's identification of *M. tuberculosis* as the cause of active tuberculosis in 1882, it was not until the 1950s that NTM were recognized to cause human pulmonary disease. Given their ubiquitous presence in the environment, it is important to distinguish colonization from active disease following isolation of NTM from pulmonary samples. In response to this challenge, the ATS/IDSA introduced stringent diagnostic criteria with clinical, radiological and microbiological components for diagnosis of pulmonary NTM disease [2].

The clinical and molecular epidemiology of prevalent NTM in low and middle-income countries, also endemic for pulmonary tuberculosis, is less known because pulmonary and other disease manifestations caused by NTM pose a diagnostic challenge to microbiologists and clinicians [2,4]. In contrast to pulmonary tuberculosis, it is not possible to readily identify pulmonary NTM disease with the usual combination of basic mycobacteriology, clinical history, radiologic imaging and the tuberculin skin test, where applicable. The culture and molecular biology identification techniques required for NTM diagnosis are not cost effective for routine clinical practice in resource-poor health systems where priority is currently given

to expanding access to diagnosis and treatment for pulmonary tuberculosis [5,6]. The distribution of NTM species isolated from pulmonary samples differs significantly by geographic region. However, most of these data are from the developed world and sub-Saharan Africa is under represented [7,8]. Although there are now emerging NTM disease data from Asia and parts of Africa, significant knowledge gaps still exist especially in sub-Saharan Africa where nine of the world's 22 high burden tuberculosis countries are found [8-11]. Therefore, fears that inconclusive diagnosis based on smear microscopy or clinical symptoms and/or radiological findings could lead to misdiagnosis of pulmonary tuberculosis and/or inappropriate management of pulmonary NTM cases are valid. As it is especially to difficult to differentiate between NTM colonisation and NTM disease the American Thoracic Society/Infectious Disease Society of America (ATS/IDSA) defined a set of clinical and microbiological criteria to diagnose pulmonary NTM disease (table 1).

The objectives of this review are to consolidate existing data on NTM colonisation and disease (according to ATS/ISDA criteria) in sub-Saharan Africa, review the existing gaps in our knowledge of pulmonary NTM and identify future research priorities.

Methods

Literature Search and Selection Criteria

This review was conducted in accordance with PRISMA guidelines [12]. The overall aim of this review was to determine the prevalence of NTM in apparently healthy and diseased individuals in sub-Saharan Africa. We defined sub-Saharan Africa as all of Africa except Northern Africa.

Search strategy

We searched PubMed, EMBASE, POPLINE, OVID and Africa Wide Information electronic databases for publications about pulmonary NTM in sub-Saharan Africa published from January 1, 1940 to October 1, 2016 using the following search terms and strategy: ((((("nontuberculous mycobacteria"[MeSH Terms] AND "africa south of the sahara"[MeSH Terms]) OR "mycobacterium infections, nontuberculous"[MeSH Terms]) AND "africa south of the sahara"[MeSH Terms]) OR "mycobacterium infections, nontuberculous"[MeSH Terms]) AND "africa south of the sahara"[MeSH Terms]) OR (("lung"[MeSH Terms] OR "lung"[All Fields] OR "pulmonary"[All Fields]) AND "nontuberculous mycobacteria"[MeSH Terms])) AND "africa south of the sahara"[MeSH Terms] AND (("1940/01/01"[PDAT]: "2016/10/01"[PDAT]) AND "humans"[MeSH Terms]).

Selection process and data abstraction

We found 373 citations from our database searches (see Fig 1). The titles and abstracts of all the articles were screened and full-text copies of those deemed relevant obtained. In addition, the reference sections of all the retrieved articles were screened to identify other eligible citations. Only articles reporting on pulmonary samples were included. For all relevant articles, we extracted the following data using a data extraction sheet: research setting, study

117 period, population tested and numbers, NTM species isolated, method for NTM
118 identification, prevalence of pulmonary NTM isolation/disease, HIV co- infection rate and
119 risk factor(s) for NTM acquisition.

120 **Data analysis**

121 In estimating country-level and overall prevalence of NTM in sub-Saharan Africa, a pooled
122 estimate was computed based on a simple meta-analysis of the reported prevalences. Each
123 study was weighted according to its sample size and the exact binomial used to derive the
124 95% confidence intervals (95% CI). We checked all retrieved articles for application of the
125 ATS/IDSA diagnostic criteria (Table 1) for clinically relevant pulmonary NTM and recorded
126 the proportion of patients meeting these criteria and NTM species responsible.

127 All extracted data were stored in Microsoft® Excel® (Microsoft Corporation, Redmond,
128 Washington, United States) and analysis carried out in STATA™ version 12.1 (College
129 Station, Texas, United States).

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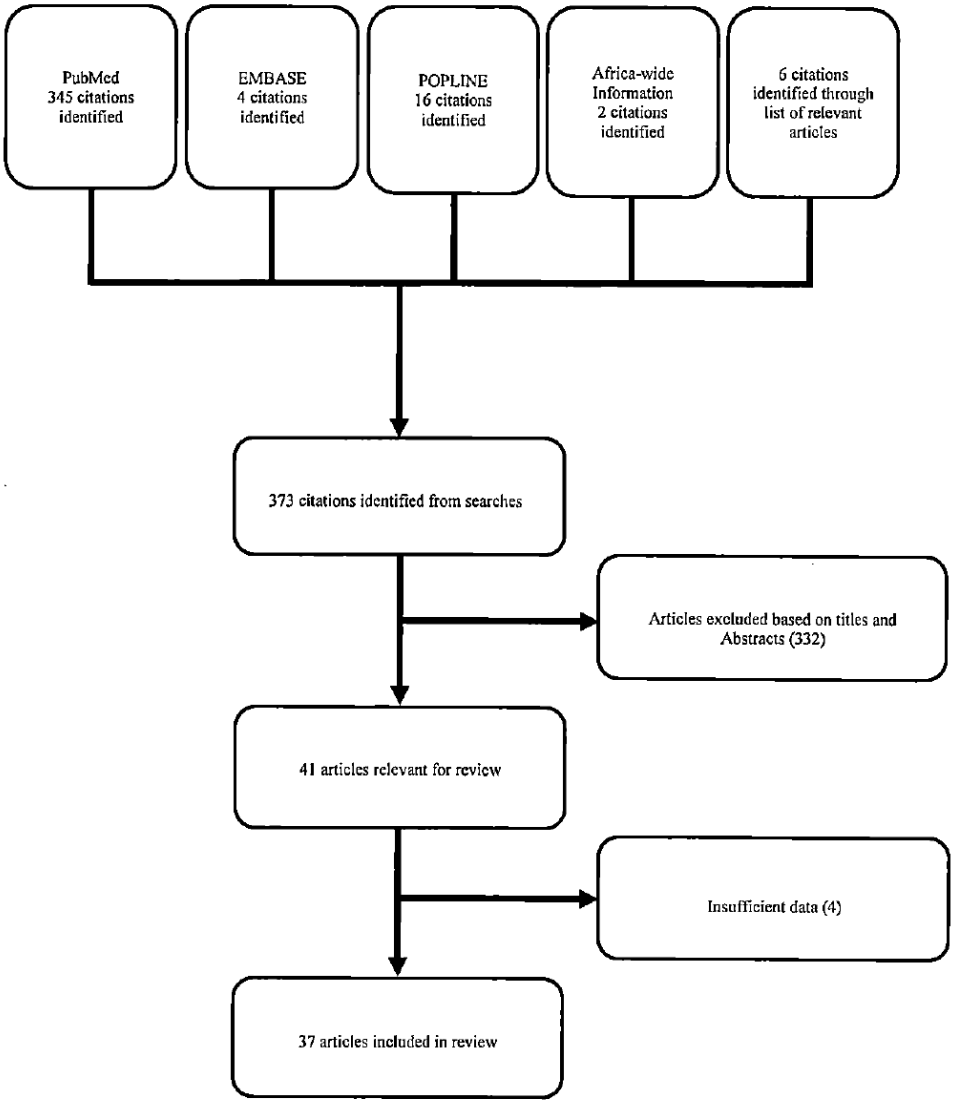

Fig 1. Flow chart of literature search and article selection criteria

Appendix

Table 1: Summary of the American Thoracic Society/Infectious Disease Society of America diagnostic criteria for pulmonary non-tuberculous mycobacterial infection/disease [2].

Clinical
1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or a high-resolution computed tomographic scan that shows multifocal bronchiectasis with multiple small nodules.
And
2. Appropriate exclusion of other diagnoses.
Microbiologic
1. Positive culture results from at least two separate expectorated sputum samples (If the results from the initial sputum samples are non-diagnostic, consider repeat sputum acid-fast bacillus (AFB) smears and cultures).
OR
2. Positive culture results from at least one bronchial wash or lavage.
OR
3. Transbronchial or other lung biopsy with mycobacterial histopathological features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathological features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.
4. Expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination.
5. Patients who are suspected of having NTM lung disease but who do not meet the diagnostic criteria should be followed until the diagnosis is firmly established or excluded.

Results

Description of included studies

There were only 37 relevant articles on the epidemiology of pulmonary NTM in sub-Saharan Africa as shown in Table 2. These were from studies in western (Nigeria, Mali and Ghana), southern (Zambia and South Africa [RSA]) and eastern (Kenya, Uganda, Tanzania and Ethiopia) Africa [5,6,8,10,13-44]. Eleven articles were from Nigeria [5,13,15-21,45,46], one from Mali [22], one from Ghana [23], six from Zambia [6,10,24-27], two from Kenya [28,29], two from Uganda [30,31], three from Tanzania [32-34], three from Ethiopia [35-37] and eight from South Africa [8,38-40,43,44].

Where methods of identification were reported, molecular techniques (n= 26) were the most frequently used to identify NTM species, followed by conventional biochemical testing identification tools (n= 9) and immunochromatographic assays (n=2). The molecular diagnostic methods used were Restriction Fragment Polymerase Chain Reaction (RFPCR) of the 65KD *hsp* gene, Genotype CM/AS assay (Hain Life science, Nehren, Germany), and *16S rRNA* gene sequencing analysis in one, eleven and fourteen studies respectively. Identification methods also varied over time and a dramatic rise in the use of molecular methods was observed in the period 2000-2016. Biochemical and phenotypic tools were the only methods used for NTM identification before 2000. Despite this transition in identification methods used over time, there was no major change in diversity of NTM species isolated in the period before and after the year 2000 as shown in Table 3.

Appendix

181 **Table 2: Overview of studies on pulmonary non-tuberculous mycobacteria in sub-Saharan Africa**

Country	Study period	Reference	Age in years	Sample size	Sputum cultures		Most isolated NTM	Method of NTM identification	Overall prevalence of NTM isolation (%)	Pulmonary NTM patients with HIV coinfection (%)	ATS/IDSA applied/numbers meeting criteria	Risk factors for pulmonary NTM
					MTBC	NTM						
Ethiopia	2010	Mathewos et al. (2015)[36]	NA	263 presumptive TB cases	110	7	NTM not classified	Immunochro-matography assay (Capilia TAUNS method)	2.7	NA	No	NA
Ethiopia	2011	Workalemahu et al. (2015)[37]	1-15	121 presumptive TB cases	15	10	*M. fortuitum* *M. parascrofulaceum* *M. triviale*	Molecular (Sequencing of *16S rRNA* gene)	8.3	NA	NA	NA
Ethiopia	2008-09	Gumi et al. (2012)[35]	NA	260 presumptive TB cases	157	7	*M. flavescens*	Molecular (Sequencing of *16S rRNA* gene)	2.7	NA	No	NA
Ghana	2013-14	Bjerrum et al. (2016)[23]	≥18	473 HIV infected adults	60	38	*M. avium* complex *M. chelonae* *M. simiae* *M. fortuitum*	Molecular (sequencing of *16S rRNA* gene)	8.0	All HIV infected	No	HIV infection and age
Kenya	2007-09	Nyamogoba et al. (2012)[28]	≥0	872 presumptive TB cases	346	15	*M. fortuitum* *M. peregrinum*	Molecular (Genotype CM/As assay)	1.7	46.7	No	Previous TB HIV infection
Kenya	2014-15	Limo et al. (2014)[29]	≥0	210 retreatment cases	121	89	*M. intracellulare* *M. abscessus* *M. fortuitum*	Molecular (Genotype CM/As assay)	42.4	25.8	No	Previous TB infection

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Mali	2004-09	Miaga et al. (2012)[22]	18-73	142 presumptive TB cases enrolled	113	17	*M. avium* *M. palustre* *M. fortuitum*	Molecular (sequencing of *16S rRNA* gene)	12.0	17.6	Yes; 11	Previous TB
Nigeria	2010-11	Olutayo et al. (2016)[13]		319 presumptive TB cases	122	26	NA	Molecular (Genotype CM/AS assay)	8.2	46.2	No	HIV infection, age
Nigeria	2008-09	Cadmus et al. (2016)[26]	NA	23 presumptive cases	11	9	*M. avium* complex	Molecular (Sequencing of *16S rRNA* gene)	39.1	NA	No	NA
Nigeria	2010-11	Gambo et al. (2014)[15]	NA	952 presumptive TB cases	254	65	NTM not classified	Molecular (Genotype CM/AS assay)	6.8	40.0	No	HIV infection, TB
Nigeria	2010-11	Gambo et al. (2013)[5]	18	1603 TB presumptive TB cases	375	69	*M. intracellulare* *M. abscessus* *M. fortuitum* *M. gordonae*	Molecular (Genotype CM/AS assay)	4.3	40.0	No	HIV infection, TB
Nigeria	2008-09	Asuquo et al. (2012)[16]	10-70	137 presumptive TB cases	81	4	*M. fortuitun* *M. avium species* *M. abscessus*	Molecular (Genotype CM/AS assay)	2.9	50.0	No	HIV infection
Nigeria	1983	Idigbe et al. (1986)[17]	NA	668 presumptive TB cases	NA	NA	*M. avium* *M. kansasii* *M. fortuitum*	Conventional biochemical methods	11.0	NA	NA	NA

Appendix

Nigeria	1982-93	Idigbe et al. (1995)[18]	NA	NA	NA	NA	*M. avium* *M. kansasii* *M. xenopi* *M. fortuitum*	Conventional biochemical methods	NA	NA	No	NA
Nigeria	NA	Mawak et al. (2006)[45]	≥14	329 presumptive cases	50	15	*M. avium* *M. kansasii* *M. fortuitum*	Conventional biochemical methods	4.6	NA	No	NA
Nigeria	2007-09	Daniel et al. (2011)[19]	25-80	102 TB patients (41 new s+ and 61s+ retreatment cases)	70	7	*M. fortuitum* *M. intracellulare* *M. chelonae*	Conventional biochemical methods	6.9	15.0	No	Previous TE
Nigeria	NA	Allana et al. (1991)[20]	NA	NA	NA	NA	*M. avium* *M. kansasii* *M. fortuitum*	Conventional biochemical methods	NA	NA	NA	NA
Nigeria	1963	Beer et al. (1965)[21]	≥1	NA	2682	149	Runyon 111 and 1V organisms	Conventional biochemical methods	6.0	NA	No	Previous TB
South Africa	2006-07	Clare et al. (2015)[38]	Media n age-44	2496 presumptive TB cases	421	232	*M. kansasii* *M. gordonae*	Conventional biochemical methods	9.3	31.9	No	HIV infectic
South Africa	1996-97	Corbett et al. (1999)[39]	NA	TB presumptive cases	NA	118	*M. kansasii* *M. fortuitum* *M. scrofulaceum*	Conventional biochemical methods	NA	34.0	Yes; 32	Previous TB silicosis
South Africa	1993-96	Corbett et al. (1999)[40]	≥18	594 mine workers	NA	406 NTM	*M. kansasii* *M. fortuitum* *M. avium* complex	Conventional biochemical methods	68.4	13.1	Yes; 206	HIV infectio silicosis

Appendix

South Africa	1993-96	Corbett et al. (1999)[39]	≥18	243 NTM infected suspects	92	243	*M. kansasii* *M. fortuitum* *M. intracellulare*	Conventional biochemical methods	100	NA	No	Previous TB, silicosis
South Africa	1993-96	Corbett et al. (1999)[45]	≥18	406 gold miners	NA	261 NTM patients	*M. kansasii* *M. scrofulaceum*	Conventional biochemical methods	64.3	NA	No	Previous TB, HIV infection
South Africa	2001-05	Hartherill et al. (2006)[43]	18 (13-23) months	1732 presumptive TB cases	94	109	*M. intracellulare* *M. gastri* *M. avium*	Molecular (RFPCR of 65 KD *hsp*gene)	6.3	4.2	Yes; 8	Previous TB
South Africa	2009	Sookan et al. (2014)[44]	NA	200 NTM suspects	NA	133 NTM patients	*M. avium* complex. *M. RGM* *M. gordonae*	Molecular (Genotype CM/AS assay)	66.5	NA	No	NA
South Africa	2008	Hoefsloot et al. (2013)[8]	NA	NA	NA	5646 NTM patients	MAC *M. kansasii* *M. scrofulaceum* *M. gordonae*	Molecular (Genotype CM/AS assay, AccuProbe assays, *hsp 65* PCR-restriction enzyme analysis, Inno-Lipa Mycobacteria and biochemical methods	NA	NA	NA	NA
Tanzania	2012-13	Hoza et al. (2016)[33]	40 7-88	372 presumptive TB cases	85	36	*M. gordonae* *M. interjectum* *M. avium* complex *M. scrofulaceum*	Molecular (Genotype CM/AS assay)	9.7	33	No	HIV infection and age
Tanzania	2011	Haraka et al. (2012)[34]	35	1 HIV negative patient with prior TB	NA	1	*M. intracellulare*	Molecular (Genotype CM/AS assay)	100	100	Yes;1	Previous TB

Appendix

Tanzania	2001-13	Katale et al. (2014)[32]	NA	472 presumptive TB cases	NA	12	*M. chelonae* *M. abscessus* *M. spaghni*	Molecular (Sequencing of *16S rRNA* gene)	2.5	NA	No	NA
Uganda	2009	Asimwe et al. (2013)[30]	12-18	2200 (710 infants and 1490 adolescents presumptive TB cases)	8	95	*M. fortuitum* *M. szulgai* *M. gordonae*	Molecular (Genotype CM/As assay)	4.3	NA	No	NA
Uganda	2012-13	Bainomugisa et al. (2013)[31]	NA	241 presumptive TB cases	95	14	*M. avium* *M. kansasii*	Molecular (Polymerase Chain Reaction of 16S rDNA using the Light cycler)	5.8	NA	No	NA
Zambia	2009-12	Mwikuma et al. (2015)[25]	NA	91 NTM suspected isolates	NA	54	*M. intracellulare* *M. lentiflavum.* *M. avium*	Molecular (Genotype CM/As assay)	59.3	NA	No	NA
Zambia	NA	Kapta et al. (2015)[24]	≥1	6123 presumptive TB cases enrolled	265	923	NTM not identified	Immunochromat ography assay (Capilia TAUNS method)	15.1	5.8	No	TB and H infection
Zambia	2001	Buijtels et al. (2010)[26]	≥15	167 chronically ill patients	74	93	*M. intracellulare* *M. lentiflavum* *M. chelonae*	Molecular (Sequencing of *16S rRNA* gene)	55.6	79.0	Yes; 7	Previous T HIV infec
Zambia	2001	Buijtels et al. (2005)[10]	≥25	4 presumptive TB cases	NA	4	*M. lentiflavum* *M. goodie*	Molecular (Sequencing of *16S rRNA* gene)	100.0	33.0	No	HIV infec damaged lungs

Appendix

	Zambia	2011-12	Malama et al. (2014)[27]	NA	100 presumptive TB cases	46	9	*M. intracellulare* *M. abscessus* *M. chimera*	Molecular (Sequencing of *16S rRNA* gene)	9.0	NA	NA	NA
	Zambia	2002-03	Buijtels et al. (2009)[6]	≥15	565 (180 chronically ill patients and 385 healthy controls)	205	93	*M. intracellulare* *M. lentiflavum.* *M. avium*	Molecular (Sequencing of *16S rRNA* gene)	16.5	45.6	Yes; 1	Previous TB HIV infection, and use of tap water

182 **NA=Data not available in article**

183 **Table 3: Non-tuberculous mycobacteria species isolated from sub-Saharan Africa, 1965-2016‡**

Non-tuberculous mycobacteria species	Prior 2010 Biochemical identification methods	After 2010 Molecular identification methods	Previously associated with disease
M. intracellulare	Y	Y	Y
M. avium	Y	Y	Y
M. kansasii	Y	Y	Y
M. chelonae	Y	Y	Y
M. abscessus	Y	Y	Y
M. fortuitum	Y	Y	Y
M. scrofulaceum	Y	Y	Y
M. lentiflavum	Y	Y	Y
M. interjectum	Y	Y	Y
M. peregrinum	Y	Y	N
M. gordonae	Y	Y	N
M. xenopi	Y	Y	Y
M. malmoense	Y	Y	Y
M. moriokaense	Y	Y	N
M. kumamotonense	N	Y	N
M. kubicae	Y	Y	N
M. gordonae	Y	Y	N
M. simiae	Y	Y	Y
M. palustre	Y	Y	Y
M. indicus pranii	N	Y	N
M. elephantis	N	Y	N
M. flavascens	Y	Y	N
M. bouchedurhonense	N	Y	N
M. chimera	N	Y	Y
M. europaeum	N	Y	N
M. neoaurum	N	Y	N
M. asiaticum	Y	Y	N
M. nonchromogenicum	N	Y	N
M. gastri	Y	Y	N

M. nebraskense	Y	Y	N
M. confluentis	Y	Y	N
M. porcinum	Y	Y	Y
M. terrae	Y	Y	N
M. seoulense	Y	Y	N
M. engbackii	Y	Y	N
M. parascrofulaceum	Y	Y	N
M. triviale	Y	Y	N
M. scrofulaceum	Y	Y	Y
M. szulgai	Y	Y	Y
M. heckeshornense	Y	Y	N
M. poriferae	Y	Y	N
M. spaghni	Y	Y	N
M. goodie	Y	Y	N
M. aurum	Y	Y	N
M. conspicum	Y	Y	N
M. mucogenicum	Y	Y	N
M. rhodesia	Y	Y	N
M. gilvum	Y	Y	N
M. genevanse	N	Y	N
M. intermidium	N	Y	N
M. fortuitum II/M. magaritense	N	Y	Y

Y=isolated N=not isolated. ‡ Data retrieved from references [5,6,8,10,16-40,43-46]

Synthesis of results

Epidemiology of Non-tuberculous Mycobacteria

The overall prevalence of NTM in pulmonary samples in sub-Saharan Africa derived from all 37 papers reviewed was 7.5% (95% CI: 7.2% - 7.8%). The median age of participants was 35 (Interquartile range, IQR 16 - 80) years based on 17 of 37 studies with age data. The majority

(2325 [75.0%] of 3096) of subjects with NTM were males. Patients in 12 of 37 studies (32.4%) had a previous history of pulmonary tuberculosis and 15 (40.5%) were co-infected with HIV.

MAC species accounted for 28.0% (95% CI: 27.2 – 28.9%) of all NTM isolated and was the most frequently encountered NTM found in pulmonary samples in 19 of 37 studies. The prevalence of MAC ranged from 15.0% (95% CI: 5.05 – 25.0%) in Tanzania to 57.8% (95% CI: 36.3 – 76.9%) in Mali as shown in Fig 2 (along with country HIV prevalence in the legend [47]). There was regional variability in the distribution of NTM for example; 76.4% (95% CI: 74.8 – 77.9%) i.e. 2,355 of 3,084 MAC isolates from South Africa were *M. intracellulare,* while all MAC isolates from Mali were *M. avium.* Similarly, while *M. kansasii* was the third most isolated NTM in sub-Saharan Africa overall (4.7% [95% CI: 4.3 – 5.1%]), it was the top NTM in five (62.5%) of eight studies in South Africa.

Other slow growing mycobacteria isolates, though less prevalent than MAC, were *M. scrofulaceum* 7.0% (95% CI: 6.4 – 7.5%) and *M. gordonae* 3.8% (95% CI: 3.4 – 4.3%). The rapidly growing mycobacteria i.e. *M. fortuitum, M. chelonae,* and *M. abscessus* accounted for just 1.2% (95% CI: 1.0 – 1.4%) of all NTM isolates from sub-Saharan Africa. Rapidly growing mycobacteria were reported predominantly from eastern African countries with *M. fortuitum* (43.0% [95% CI: 34.4 – 53.2%]) and *M. abscessus* (16.0% [95% CI: 9.4 – 25.9%]) as the top and second ranked NTM isolates from Uganda and Kenya respectively.

Among the 70.8% (6357 of 8980) fully speciated isolates in this review, there were 0.9% (56) *M. lentiflavum,* 0.9% (55) *M. malmoense,* 0.7% (43) *M. xenopi,* 0.4% (24) *M. gastri,* 0.3% (16) *M. szulgai,* 0.2% (15) *M. flavescens,* and 0.2% (11) *M. interjectum.* Unfortunately, 29.2% (95% CI: 28.1 – 30.1%) i.e. 2,623 of all 8,980 NTM isolates were not identified to species level.

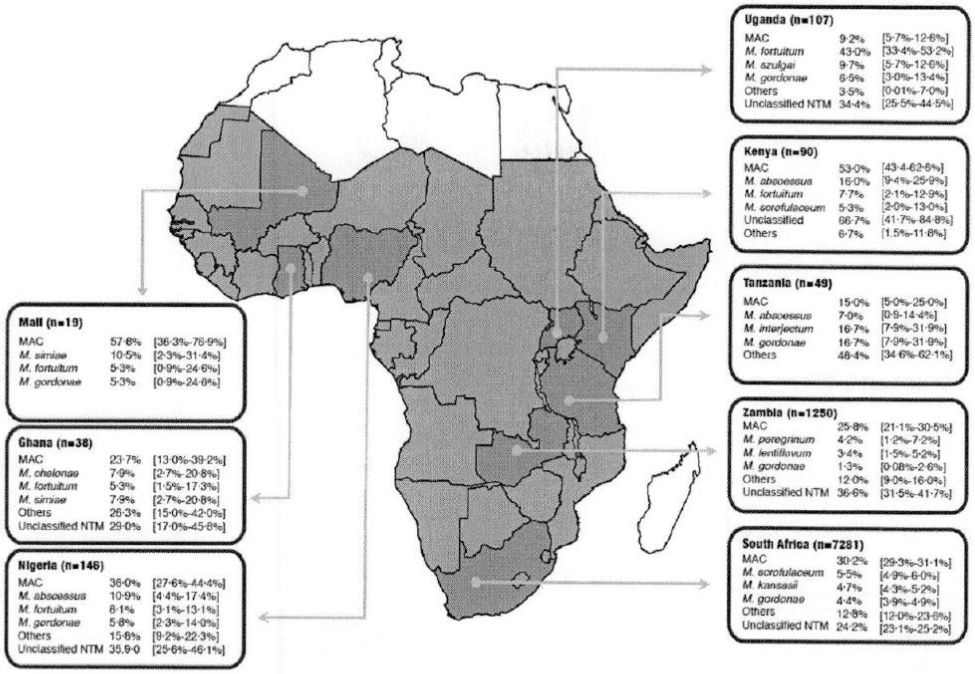


Fig 2. The distribution of the top four non-tuberculous mycobacteria species identified from pulmonary samples in Mali (HIV 1.4%), Ghana (HIV prevalence 1.3), Nigeria (HIV 3.1%), Uganda (HIV 7.1%), Kenya (HIV 5.9%), Tanzania (HIV 4.7%), Zambia (HIV 12.9%), and Republic of South Arica (HIV 19.2%), without considering clinical relevance. Data compiled from references [5,6,8,10,13,15-17,19-33,35-46]. HIV prevalence compiled from reference [47].

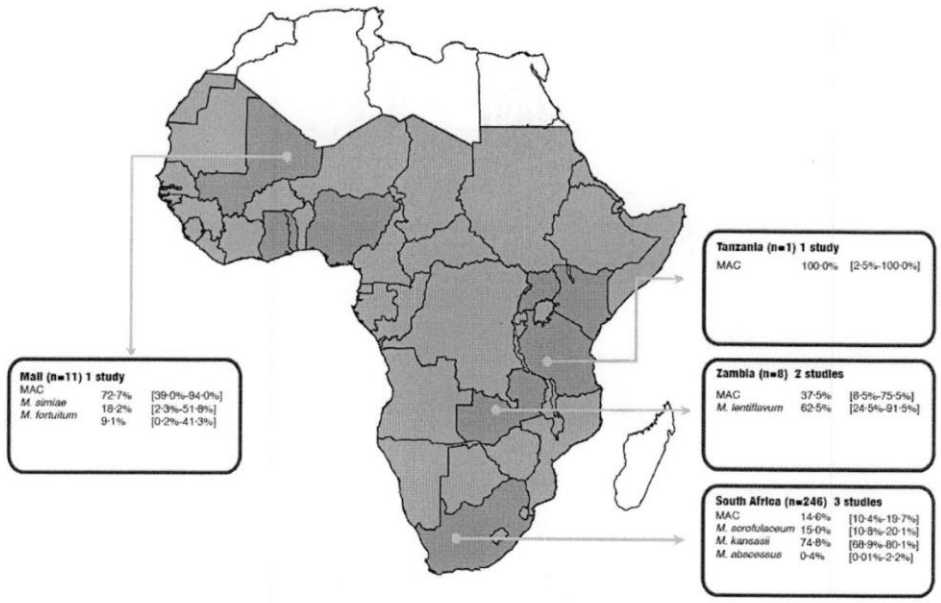
Map was created using Adobe Illustrator version CS6 (Adobe Systems)

Epidemiology of Pulmonary Non-tuberculous Mycobacterial Disease

One particular challenge in studying NTM infection is the difficulty in differentiating between NTM colonisation of patients (due to the mere presence of the bacteria in the environment) and actual pulmonary disease. Therefore the American Thoracic Society/Infectious Disease Society of America (ATS/IDSA) defined a combination of stringent clinical and microbiological criteria to conclusively determine pulmonary disease (see table 1). To evaluate the geographical distribution of disease-causing NTM only, we excluded 30 articles that only reported on the detection of NTM without applying ATS/IDSA criteria and therefore could not show evidence of pulmonary disease. Only seven (19.0%) of the 37 articles were ATS/ISDA compliant and could be investigated in respect to the epidemiology of clinically relevant NTM [6,22,26,34,39,40,43]. Although these studies had 3,319 participants, only 962 (28.9%) had sufficient information to apply the ATS/IDSA criteria and of these, 266 (27.7%) met the definition of pulmonary NTM disease. *M. kansasii,* isolated in 184 (69.2%) of 266 cases, was the most predominant cause of confirmed pulmonary NTM disease, followed by *M. scrofulaceum* (13.9%), MAC (13.5%), *M. lentiflavum* (1.9%), *M. simiae* (0.8%), *M. palustre* (0.4%) and *M. abscessus* (0.4%).

Fig 3 shows the distribution of NTM species causing pulmonary NTM disease in sub-Saharan Africa by country. The studies investigating the clinical relevance of NTM isolates varied substantially in design, participant characteristics and background HIV prevalence (see Table 2). They ranged from a Zambian study that evaluated the clinical relevance of NTM isolated from 180 chronically ill patients and 385 healthy controls and found only 1.1% of isolates were clinically relevant [6], to a Malian study in patients with primary and chronic pulmonary tuberculosis where 57.9% of isolated NTM were clinically relevant [22].

248



249

250 **Fig 3. Non-tuberculous mycobacteria species causing pulmonary disease (based on ATS/ISDA criteria)**

251 **found in respiratory specimens in sub-Saharan Africa. Data compiled from references [6,22,26,34,39,40,43].**

252 **Map was created using Adobe Illustrator version CS6 (Adobe Systems)**

253

Clinical and Radiological Signs, and Associated Morbidities

Of 3096 participants with NTM isolates, 80.7% (2498) and 87.5% (2,709) had clinical and radiological information respectively [5,6,15,16,21,22,24-26,29,34,38-40,43,45]. Clinical characteristics for NTM subjects closely mimicked those of pulmonary tuberculosis, as summarized in Table 4. There were radiological abnormalities in 79.0% (2141) of 2709 subjects, while 21.0% (568) had normal chest radiographs. Of the 512 with prior lung disease, 87.1% (446) had a history of tuberculosis and 12.9% (66) had bronchiectasis. In those with concurrent conditions, 50.2% (442) of 880 were coinfected with HIV, 28.2% (248) reported gastrointestinal diseases and 8.6% (76) complained of body weakness. Other characteristics are shown on Table 4.

Table 4: Clinical and radiographic characteristics for patients with pulmonary non-tuberculous mycobacteria infections in sub-Saharan Africa, 1965-2016 (N= 3096)

Characteristic	Numbers (%)
Clinical signs n=2, 498	
Cough ≥2 weeks	950 (38.0%)
Chest pain	684 (27.3%)
Significant weight loss	546 (21.9%)
Fever≥2	455 (18.2%)
Night sweats	211(8.4%)
Haemoptysis	27 (1.1%)
Dyspnoea	19 (0.8%)
Previous lung disease n= 512	
Bronchiectasis	66 (12.9%)
Tuberculosis	446 (87.1%)
Radiographic findings n= 2709	
Abnormal, suggestive of TB	1009 (37.2%)
No pathological changes	568 (20.9%)

Tuberculosis	446 (16.5%)
Nodules	203 (7.5%)
Fibrosis	140 (5.2%)
Cavitation	127 (4.7%)
Prior focal radiological scarring	107 (4.0%)
Bronchiectasis	66 (2.4%)
Abnormal, not consistent with TB	24 (0.9%)
Milliary TB	19 (0.7%)
Concurrent conditions n=880	
HIV infection	442 (50.2%)
Gastrointestinal disease	248 (28.2%)
Weakness	76 (8.6%)
Lymph node enlargement	52 (6.0%)
Splenomegaly	21 (2.4%)
Diabetes mellitus	22 (2.5%)
Hepatomegaly	19 (2.2%)

266

Discussion

We provide an overview of the epidemiology and geographical distribution of NTM species isolated from pulmonary samples in sub-Saharan Africa. To our knowledge, this is the first comprehensive review of pulmonary NTM in this part of the world. Similar to reviews by other authors, our findings suggest diversity in prevalent NTM species, geographical variation in NTM distribution and their clinical relevance across the sub-continent [48].

The global collection of NTM isolated from pulmonary samples reported by Hoefsloot et al. [8] in 2008 included isolates from only one sub-Saharan Africa country, South Africa. The update in 2013 by Kendall et al. did not improve significantly on the earlier review with respect to additional African NTM isolates [1]. Despite the relative scarcity of local data, it is important to highlight that this review is the first to include NTM data for nine countries in sub Saharan Africa.

Overall, we found a predominance of MAC from pulmonary samples in countries of Western, Eastern and Southern Africa. *M. scrofulaceum* and *M. kansasii* were predominant in Southern Africa and the rapidly growing mycobacteria (*M. abscessus*, *M. fortuitum* and *M. chelonae*) in Eastern Africa. These findings are consistent with the predominance of MAC in the epidemiology of NTM in North America [1,2,49], Europe [50], Australia [51] and East Asia [52]. The relative preponderance of the two members of the MAC family also varied by region with *M. intracellulare* predominating in South Africa while all MAC isolates from Mali were *M. avium*. However, the South African study had a much bigger sample size compared to the Malian study. While MAC was the most frequently implicated NTM in colonisation, *M. kansasii* was the most common in pulmonary NTM disease. The dominance of *M. kansasii* as well as the presence of *M. scrofulaceum* in South Africa was speculated to be linked to mining activities and significant urbanisation in the country, resulting in a socio-

291 economically disadvantaged population [7,53,54], working in the mines, frequently suffering
292 from silicosis, while living in poor, overcrowded environments (also see Table 2). When the
293 South Africa pulmonary NTM data is excluded, MAC is the major cause of pulmonary NTM
294 disease as reported in North America, Europe, Australia and Asia [1]. Because relatively few
295 studies in this review applied the ATS/IDSA criteria for confirmation of pulmonary NTM
296 disease, it is difficult to reach conclusions regarding the dominant NTM species causing
297 pulmonary disease in sub-Saharan Africa.

298 The reason for the observed geographical variation in NTM populations across Africa is still
299 unknown, and could be due to environmental factors associated with the differing
300 geographical country locations. Unfortunately included studies were not designed to
301 investigate sub-regional geographical variation and did not systematically collect
302 environmental data. Ideally future studies on NTM in Africa could address this issue.

303 In contrast to observations from other parts of the world, especially in Europe, where *M.*
304 *malmoense* and *M. xenopi* are well known for causing pulmonary NTM disease [1,55,56], these
305 NTM were not represented in the limited number of studies reviewed here. *M. xenopi* was
306 rare in sub-Saharan Africa, which is not unexpected considering its association with hot water
307 delivery systems that are less developed in sub-Saharan Africa compared to industrialised
308 countries [2,57].

309 Pulmonary NTM was commonly associated with a history of previous pulmonary
310 tuberculosis in sub-Saharan Africa compared to Europe and North America. This is not
311 surprising given the high incidence of MTBC disease in sub-Saharan Africa [58,59]. Pulmonary
312 tuberculosis is associated with significant sequelae that have not been adequately studied in
313 sub-Saharan Africa. The associated structural lung damage, chronic pulmonary obstructive
314 disease and infections most likely favour colonization by NTM and other pathogens [60]. It is

315 also likely that the increasing isolation of NTM has come from investigation of patients with
316 chronic pulmonary disease including those complicating previous pulmonary tuberculosis [6,22].
317 In light of this, the clinical, radiological and microbiologic criteria of the ATS\IDSA is
318 important for distinguishing colonization from pulmonary NTM, particularly in sub-Saharan
319 Africa that is endemic for MTBC [61].

320 Many rarely isolated NTM were also identified in presumptive tuberculosis patients, for
321 example *M. genavense*, *M. gilvum*, *M. intermedium*, *M. poriferae*, *M. spaghni, M.*
322 *interjectum, M. peregrinum, M. moriokaense, M. kumamotonense and M. kubicae.* Although
323 some of these species have also been isolated in other parts of the world from pulmonary
324 samples in patients with chronic bronchitis, pulmonary tuberculosis, sub-acute pneumonia
325 and healed tuberculosis [62,63], it is currently unclear what role they play in the aetiology of
326 pulmonary disease in Africa.

327 The HIV-driven increase in the risk of tuberculosis disease in sub-Saharan Africa has been
328 well described and for NTM, MAC is a particularly well described opportunistic infection in
329 patients with AIDS. We found almost half of all cases of confirmed pulmonary NTM were
330 also HIV co-infected. This suggests the possibility of HIV attributable pulmonary NTM
331 beyond the now familiar disseminated MAC disease often seen in persons with AIDS.

332 Persons with pulmonary NTM infection in sub-Saharan Africa are younger than observed in
333 North America, Europe and Australia where increasing age (≥50 years), structural lung
334 damage, immunosuppressive chemotherapy for cancer, autoimmune and rheumatoid
335 conditions are the most frequently reported risk factors for this disease [1,2,60,64]. Given the
336 younger age and higher burden of pulmonary tuberculosis and HIV co-infection in sub-
337 Saharan Africa, it is not surprising that we found pulmonary NTM infection mostly in the 33-
338 44 year-age group. As the ATS/ISDA compliant studies did not describe the clinical

characteristics of individual NTM patients, a risk-factor analysis for NTM disease could not be conducted in the present review.

Our review has a number of limitations: we only searched for English language-articles. Given the numbers of Francophone countries in sub-Saharan Africa, French-language publications may have been missed. In addition, our assessment of the clinical relevance of isolated NTM was not as comprehensive as desired because the majority of the studies did not collect the detailed clinical, radiological and microbiological data required to do this. We also could not report the full diversity of NTM in colonization and disease because almost 30% of all isolates were not fully identified to species level. Since the studies reviewed came from varied time periods during which laboratory procedures for ascertainment differed, we cannot exclude the possibility of laboratory procedures before and/or after year 2000 selecting for particular NTM species whilst inhibiting others [65]. For example, the wider usage of sensitive liquid culture media could in theory have selected for specific NTM species. Similarly, the increasing use of molecular methods for identification of current and historical isolates, especially for the MAC and rapidly growing mycobacteria groups, could underpin the changes to NTM taxonomy over time [66-68]. However, we think our results were not significantly affected because the distribution of NTM species identified in the periods before and after 2010 were similar. Given the heterogeneity of studies included in this review including laboratory methods and quality standards, some of the NTM reported here may be due to contamination especially for NTM like *M.flavescens* that are frequent laboratory contaminants. It is possible for example that all seven *M. flavescens* are contaminants. In more than half of 26 studies that used molecular techniques to identify NTM, 16s rDNA sequencing was used. However, this method has a limitation in that it is not fully capable of distinguishing between all the different NTM species for example *M. abscessus* and *M.*

chelonae. Therefore, it is possible some species have been misidentified or misclassified in these studies.

To conclude, we have provided the first detailed review of pulmonary NTM in sub-Saharan Africa and highlight the contribution of NTM to the aetiology of tuberculous-like pulmonary disease in the sub-continent. Our review also suggests that the presence of NTM as commensals in pulmonary samples may confound the diagnosis of pulmonary tuberculosis, especially in those with a previous history of tuberculosis and/or other chronic respiratory conditions.

Additional research and surveillance is required for investigation of the full contribution of NTM to pulmonary disease, to describe the full repertoire of prevalent and incident NTM, and to determine the role of risk factors (particularly HIV/AIDS) for colonization and/or disease. Given the risk of over diagnosis of NTM in pulmonary samples as tuberculosis disease, resulting in repeated courses of treatment in previously treated tuberculosis patients, investments in, and development of, point of care diagnostics for NTM are required.

Evidence before this Study

We searched PubMEd, Embase and other databases for the terms "nontuberculous mycobacteria*", "pulmonary*", "africa south of the sahara*", "lung", and "human". We searched for English-language articles published up to Oct 1, 2016 and reviewed all eligible articles and their reference lists. Earlier reviews only included NTM isolates, subject level data from just one sub-Saharan Africa country and did not investigate the clinical relevance of isolated NTM.

Added Value of this Study

This is the first review to utilise all available data to provide a detailed picture of the clinical and molecular epidemiology of NTM isolated from pulmonary samples in sub-Saharan Africa. As a result, we find there is a substantial burden of pulmonary NTM in the sub-continent. With seven out of every 100 presumptive tuberculosis cases either colonised or diagnosed with confirmed pulmonary NTM, the likelihood of pulmonary tuberculosis over diagnosis especially in those with previous history of tuberculosis requires further investigation. In addition, we highlight the knowledge gap resulting from incomplete identification of NTM species.

Author Contributions

CO led data acquisition from relevant articles and analysis and wrote the first draft. SM contributed in data analysis. MA, SA, FG, and IA provided supervision, and contributed to analysis and interpretation of data. FG and IA contributed equally. IA conceived the idea along with MA. All authors were involved in writing this manuscript and gave approval for publication.

Competing interest

The authors declare that they have no competing interest.

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